Rare coagulation disorders

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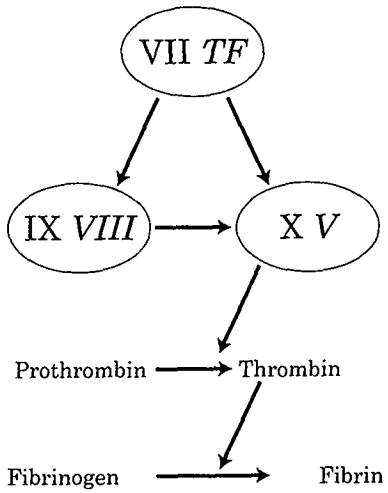
Chapter 3. **Molecular studies**

chapter 1

Introduction

Blood coagulation consists of a series of reactions that ultimately lead to the formation of the proteolytic enzyme thrombin that transforms soluble fibrinogen into three-dimensional fibrin (Figure 1). Inherited deficiencies or defects of plasma proteins involved in blood coagulation generally lead to lifelong bleeding disorders, whose severity is inversely proportional to the degree of factor deficiency (less factor/more bleeding). The most frequent are hemophilia A and B, due to the deficiency or defect of factor VIII and IX respectively. Inherited as X-linked recessive traits, hemophilia A and B have prevalence in the general population of approximately 1 in 10,000 and 1 in 50,000 with no significant racial difference (1). Hemophilia A and B are clinically indistinguishable from each other and occur in mild, moderate and severe forms (corresponding to plasma factor levels of 6-30%, 2-5% and 1% or less, respectively). Other inherited defects of coagulation factors that cause a bleeding disorder (afibrinogenemia, hypoprothrombinemia, deficiencies of factors V, VII, X, XI and XIII) are generally much rarer. They have prevalences varying between 1 in 500,000 and 1 in 2,000,000 (Table 1), with the exception of factor XI deficiency which reaches polymorphic frequency among Ashkenazi Jews and von Willebrand disease which may affect 1 in 100 (2). All these defects are transmitted as autosomal recessive traits expressed clinically only in homozygotes. As a consequence of the rarity of these disorders, the type and severity of symptoms, the spectrum of gene defects and the management of bleeding episodes are not so known and established as for hemophilia A and B and von Willebrand disease.

Fig. 1 - **A simplified scheme of blood coagulation**



The oval represent a surface of procoagulant phospholipid, clotting factors are indicated by roman numerals, the (pro-) enzymes are underlined, the cofactors are in italics.
TF= tissue factor
The feed back activation of factor V and VII by thrombin is not indicated.

In populations where consanguineous marriages are frequent, such as those from Muslim countries and Southern India, recessive coagulation disorders are more frequent and reach together prevalences higher than those of hemophilia B, representing a significant clinical and social problem. This situation typically occurs in the Islamic Republic of Iran, a country with a population of 64

Table 1 - **General features of rare coagulation disorders**

Deficiency	Estimated prevalence	Approximate number of reported cases	Gene on chromosome	Approximate number of reported mutations*
Fibrinogen	**1:1 million**	**300**	**4**	**< 90***
Prothrombin	**1:2 millions**	**40**	**11**	**< 20**
Factor V	**1:1 million**	**150**	**1**	**< 10**
Combined factor V+VIII	**1:1 million**	**40**	**18**	**< 6**
Factor VII	**1:500,000**	**150**	**13**	**< 80**
Factor X	**1:1 million**	**40**	**13**	**< 25**
Factor XIII	**1:1 million**	**60**	**A subunit:6**	
			B subunit:1	**< 20**

* *mainly in dysfibrinogenemia*

millions, where a registry of congenital coagulation disorders has been kept since the 1970s (3). Table 2 compares the absolute number and prevalences of registered Iranian patients with clinically significant inherited coagulation disorders (factor levels of 10 per cent or less) with those registered in the United Kingdom by the Hemophilia Center Director Organiza-tion (UKHCDO) and in Italy by the Istituto Superiore di Sanità on behalf of the Associazione Italiana Centri Emofilia (AICE). The three countries are comparable not only because they have similar general populations of approximately 60 million, but also because they have kept hemophilia registries for a long time. Table 2 shows that while in the three countries the total numbers of registered patients are similar, the number and prevalences of inherited deficiencies of fibrinogen, prothrombin, factor V, factor V and VIII, factor VII, factor X and factor XIII are three to seven times higher in Iran. Only factor XI deficiency is more prevalent in the United Kingdom than in Iran and Italy, probably because Jewish communities are relatively small in the latter countries. With this as background, a joint project was initiated in 1996 between the Tehran Hemophilia Center and those in London and Milan. Our plans were to unravel the molecular basis of rare coagulation disorders and, taking advantage of a large and regularly followed patient population, to establish the pattern of bleeding symptoms and identify the most efficacious and safe forms of treatment. The purpose of this thesis is to summarize the general features of recessive coagulation disorder as known hitherto, to review the experience gained in a large series of patients in terms of clinical manifestations and treatment and to characterize the molecular defects underlying those disorders.

Factor XIII deficiency and type 3 von Willebrand disease will not be dealt with because data collection and genotypic analysis on patients with these defects is still incomplete.

Table 2 - **Number of patients and (between parenthesis) relative frequency of inherited coagulation disorders in Iran, Italy and United Kingdom (excluding von Willebrand disease).**

Defect	Iran	Italy	United Kingdom
Fibrinogen	70 (1.5%)	10 (0.2%)	11 (0.2%)
Prothrombin	15 (0.3%)	7 (0.2%)	1 (0.02%)
Factor V	70 (1.5%)	21 (0.5%)	28 (0.6%)
Factor VII	300 (6.6%)	58 (1.3%)	62 (1.3%)
Factor V+VIII	80 (1.7%)	29 (0.7%)	18 (0.3%)
Factor VIII (hemophilia A)	3000 (65.4%)	3428 (80.0%)	3554 (76.8%)
Factor IX (hemophilia B)	900 (19.6%)	626 (15.0%)	762 (16.1%)
Factor X	60 (1.3%)	16 (0.4%)	25 (0.5%)
Factor XI	20 (0.4%)	60 (1.3%)	150 (3.3%)
Factor XIII	80 (1.7%)	31 (0.7%)	26 (0.5%)
All defects	4595	4286	4637

Data are obtained from the most recent adjournments (1996) of the Registries of Inherited Bleeding Disorders kept in Iran (courtesy of Mr. Afshar and Dr. Lak, Iman Khomeini Hospital and Iranian Hemophilia Society), Italy (Dr. A. Ghirardini, Istituto Superiore di Sanità) and United Kingdom (Dr. P. Giangrande, UK Hemophilia Center Director Organization). Only patients with factor levels of 10 per cent or less were evaluated (for fibrinogen deficiency, 10 mg/dL or less).

1.1 Fibrinogen deficiency

Fibrinogen deficiency is heterogeneous and two main phenotypes can be distinguished. In afibrinogenemia, plasma levels of the protein are unmeasurable or very low using assays measuring clottable and immunoreactive protein whereas in dysfibrinogenemia low clottable fibrinogen contrasts with normal fibrinogen antigen. Three separate genes located on chromosome 4 code for the α-, β- and γ- chains of fibrinogen (1). There is at the moment very little information on the gene lesions that cause afibrinogenemia (4,5), whereas more than 80 abnormalities associated with dysfibrinogenemia have been identified (6). Experimental disruption of the α-chain gene made mice completely deficient in all the fibrinogen chains (7). There was no evidence of defective embryonal development but overt bleeding developed at birth in about one third of the animals, most frequently in the peritoneal cavity, skin and joints. Ultimately blood loss was controlled, so that most mice survived the neonatal period and reached adulthood despite recurrent bleeding episodes (7).

1.2 Prothrombin deficiency

Prothrombin deficiency is probably the rarest coagulation defect, with a prevalence of 1 in 2,000,000. Measuring plasma levels of prothrombin as functional activity or immunoreactive protein, two main phenotypes can be distinguished: hypoprothrombinemia, characterized by concomitantly low levels of activity and antigen, and dysprothrombinemia, characterized by the normal synthesis of a dysfunctional protein (low coagulant activity but normal antigen levels) (8,9). To our knowledge no living patient with unmeasurable plasma prothrombin has been reported so far, consistent with experimental data indicating

that in mice complete prothrombin deficiency is incompatible with life (10). Prothrombin is encoded by a gene located in chromosome 11 (1). There is at the moment relatively little published information on the molecular basis of hypoprothrombinemia and dysprothrombinemia. It is remarkable that only gene mutations causing single amino acid substitutions have been identified so far in patients with hypoprothrombinemia (11).

1.3 Factor V deficiency

The majority of cases are characterized by the concomitant deficiency of factor V activity and antigen, but approximately one fourth have normal antigen levels, indicating the presence of a dysfunctional protein (12). Factor V is contained in platelets, probably synthesized by mega-karyocytes (13). About one third of patients with inherited factor V deficiency have a prolonged bleeding time (11). The relationship between this abnormality of primary hemostasis and the content and function of factor V in patient platelets remains unclear. The factor V gene is on chromosome 1, and a relatively small number of the molecular lesions which underlie the deficiency has been identified (14-17). The experimental deficiency of factor V in mice leads to a dramatic bleeding tendency with defective embryonic development and early hemorrhagic death (18). However, a more recent study has been shown that mice that express minimal factor V activity below the levels of assay detection (<0.1%) differ from the original knock out mice because they survive (19).

1.4 Factor VII deficiency

Factor VII deficiency is the most common of the rare coagulation disorders (1 per 500,000 population) (1). The majority of patients have concomitantly low levels of factor VII functional activity and antigen, but some cases are characterized by normal or low borderline levels of factor VII antigen contrasting with lower levels of functional activity (20). In some patients factor VII coagulant activity is highly dependent upon the source of tissue factor used in the assay (20). The factor VII gene is on chromosome 13 adjacent to the factor X gene (1), and at least 80 mutations have been identified in deficient kindreds (21). A list of these mutations can be obtained in the Internet http://europium.mrc.rpms.ac.uk/usr/WWW/WebPages/FVII/database.dir/titlespage.htm . Most mice made experimentally deficient in factor VII develop normally but some suffer fatal perinatal bleeding (22).

1.5 Combined deficiency of factor V and factor VIII

For many years the mechanism of the combined deficiency of these clotting factors coded by different genes and transmitted with different patterns of inheritance (autosomal recessive for factor V, X-linked for factor VIII) has not been understood. Recently, a substantial proportion of patients were found to have mutations in the gene ERGIC-53 on chromosome 18 (23). This gene encodes a 53 kD homo-hexameric transmembrane lectin which resides in the endoplasmic reticulum/Golgi intermediate

compartment (ERGIC) and probably acts as a chaperone in the intracellular transport and secretion of both factor V and factor VIII (24). Efficient trafficking of factor V and VIII requires a functional ERGIC and is dependent on post-translation modifications of a specific cluster of aspargic acid linked oligosaccharides to a fully glucose trimmed mamose structure. Patients with the combined defect of factor V and factor VIII have concomitantly low levels of the two factors (usually, between 5 and 20%), both as coagulant activity and antigen (25). Combined defects of clotting factors other than factor V and VIII have been described in very rare cases (26).

1.6 Factor X deficiency

Like factor VII, factor X is encoded by a gene on chromosome 13 (1). No more than 25 mutations associated with factor X deficiency have been described so far (for reviews, see 27,28). Phenotypes are characterized by concomitantly low levels of coagulant activity and antigen or by low coagulant activity contrasting with normal or low borderline antigen values. Mice rendered experimentally deficient in factor X by targeted inactivation of the factor X gene showed frequent embryonic lethality (29). Those who survived bled to death intra-abdominally at birth and in the central nervous system within the first 3-4 weeks of life (29).

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chapter 2

Clinical studies

Bleeding and thrombosis in 55 patients with inherited afibrinogenemia
British Journal of Haematology 107: 204-206, 1999

Symptoms of inherited factor V deficiency in 35 Iranian patients
British Journal of Haematology 103: 1067-1069, 1998

Clinical manifestations in 28 Iranian and Italian patients with severe factor VII deficency
Haemophilia 3: 242-246, 1997

Bleeding symptoms in 27 Iranian patients with factor V and VIII combined deficiency
British Journal of Haematology 100: 773-776, 1998

Congenital factor X deficiency: spectrum of bleeding symptoms in 32 Iranian patients
British Journal of Haematology 102: 626-628, 1998

Introduction

Textbooks contain chapters on rare coagulation disorders and describe their clinical manifestations (1,2). A review article was published in 1985 (3). In these sources, information on the spectrum of bleeding symptoms stems from reports of single cases or small series of patients. There have been attempts to analyze a larger number of cases by pooling those reported in the literature (3) or distributing questionnaires to the largest hemophilia centers (4). Because these approaches have obvious limits of inaccuracy, incomplete ascertainment and bias towards publishing the most striking cases, in this thesis we chose to minimize these drawbacks by directly investigating a relatively large number of patients from the Iranian registry. For factor V, factor VII and factor X deficiencies approximately three-fourth of the patients had a severe deficiency (plasma levels of 1% of normal or less). The remaining patients with these defects had moderately severe (2-5%) or mild deficiencies (6-10%). No patient with factor levels higher than 10% was considered, except for combined factor V and VIII deficiency (factor ranges 4-14%). None of the patients with prothrombin deficiency had factor levels below 4%. All patients with fibrinogen deficiency had levels below 10 mg/dL, which is the lower limits of sensitivity of the functional assay used in this study. Therefore, it appears that the different groups of patients with factor deficiencies were comparable, with a prevalence of severe defects except for prothrombin and combined factor V and VIII deficiency.

To evaluate critically the reliability of bleeding symptoms as reported by patients, a few criteria were established before the study in order to evaluate whether or not symptoms had to be accepted as valid. Epistaxis qualified only when it occurred spontaneously more than five times lifelong, from both nostrils, lasted more than 10 min or required hospital admission. Menorrhagia was defined as menstrual periods lasting at least 6 days and requiring the therapeutic use of combined estrogen-progestogen preparations or causing iron deficiency. Bleeding in the gastrointestinal and urinary tracts and in the central nervous system had to be documented by hospital records. Muscle hematomas and hemarthroses qualified when they occurred spontaneously or following minor traumas and caused at least transient signs of functional joint or muscle impairment. Oral bleeding qualified if it lasted more than 10 min or required the intervention of an oral surgeon, whether caused by dental extractions or by bites to lips, cheecks and tongue. Surgical or postpartum bleeding had to cause a delay in discharge from hospital or require blood transfusion. Bleeding symptoms occurring after dental extractions or surgery (inclu-

ding circumcision) qualified only when they occurred before the coagulation defect was diagnosed, in patients who had not received replacement therapy before the procedures. Easy bruising was not considered because the evaluation of this symptom by patients was considered too subjective. Clinical histories were collected by the same physician for all patients. The next articles included in this thesis report the data obtained in patients with afibrinogenemia (2.1), factor V deficiency (2.2), factor VII deficiency (2.3), combined factor V and factor VIII deficiency (2.4) and factor X deficiency (2.5).

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Bleeding and thrombosis in 55 patients with inherited afibrinogenemia

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Summary

Knowledge on the spectrum of symptoms in patients with inherited afibrinogenemia is limited by the rarity of this coagulation defect. We compared a large series of 55 afibrinogenemic patients from Iran with 100 patients with severe factor VIII deficiency, the most frequent inherited defect of coagulation. In afibrinogenemia there was a higher frequency of mucosal-type bleeding symptoms but joint and muscle bleeding was less frequent and severe than in hemophilia. Umbilical cord bleeding was relatively frequent only in afibrinogenemic patients. Two young patients developed spontaneous thrombotic episodes and three women had recurrent abortions. Overall, in afibrinogenemia bleeding symptoms are qualitatively different and somewhat less severe than in hemophilia. Afibrinogenemia can also be accompanied by thrombotic manifestations.

Keywords: afibrinogenemia, thrombosis, hemophilia

Introduction

Afibrinogenemia, a rare coagulation disorder transmitted as an autosomal recessive trait (Roberts & Bingham, 1998), is relatively more frequent in communities where consanguineous marriages are common (Fried & Kaufman, 1980). In Iran a national registry of inherited coagulation disorders lists 70 patients with afibrinogenemia. In contrast, similar registries kept in Italy and in the United Kingdom, countries that like Iran have general populations of approximately 60 millions but relatively few consanguineous marriages, list 10 and 11 patients, respectively. Knowledge on the overall spectrum of bleeding manifestations in afibrinogenemia are scanty and probably inaccurate, being based upon reports of single cases or small series that tend to emphasize the most severe and striking symptoms (Fried & Kaufman, 1980; Al-Mondhiry & Ehmann, 1994). Thrombotic complications have been described in afibrinogenemic patients (Roberts & Bingham, 1998). In this study, we tried to provide more accurate information by examining a large sample of 55 Iranian patients. Their symptoms were compared with those observed in patients with severe hemophilia A, the most typical and frequent inherited disorder of blood coagulation.

Patients and methods

Afibrinogenemia was diagnosed when plasma fibrinogen levels were unmeasurable both as functional activity (the Clauss method based upon the fibrin polymerization time using bovine thrombin; Laboratoire Stago, Asnieres, France) and immunoreactive protein (electroimmunoassay using rabbit anti-human fibrinogen antiserum supplied by Laboratoire Stago). Standards were supplied by the manufacturer. The lower limit of sensitivity of both assays is 10 mg/dL. Factor VIII deficiency was diagnosed by an APTT-based assay using substrate plasma from a severely deficient patient.

Fifty-five patients from 48 kindreds (2-73 years, 27 males and 28 women, 78% of all registered patients) were examined in

Tehran by the same physician (M.L.). Most of them were from consanguineous marriages and the asymptomatic parents had in average half-normal levels of plasma fibrinogen. To evaluate critically the reliability of bleeding symptoms as reported by patients qualification criteria were established (Lak et al, 1998). In any give patients, muscle hematomas and hemarthroses were considered as symptoms when they occurred spontaneously or disproportionately to minor traumas and caused at least transient clinical signs of functional impairment of the involved joints or muscles. Thrombotic episodes and life-endangering hemorrhagic symptoms such as umbilical cord, gastrointestinal, urinary tract and central nervous system bleeding had to be documented by hospital records. A mild mucosal tract hemorrhage such as epistaxis qualified only when it occurred more than five times lifelong, from both nostrils, lasted more than 10 min or required hospital admission. Menorrhagia, evaluated in 20 women of childbearing age, was defined by menstrual periods that lasted at least 6 days and required treatment (replacement therapy, antifibrinolytic amino acids, estrogen-progestogen medication or iron supplementations). Oral bleeding qualified if it did require local hemostasis measures, whether caused by dental extractions or fall of deciduous teeth or by bites to lips, cheeks and tongue. Excessive bleeding after surgery (including circumcision) and in the post-partum period qualified only when it occurred before the diagnosis of afibrinogenemia was established, so that no prophylactic replacement therapy had been administered.

Table 1 - **Relative frequency of bleeding symptoms in 55 patients with afibrinogenemia compared with 100 patients with severe hemophilia A (factor VIII 1% or less)**

Symptom	In afibrinogenemia	In Hemophilia A
Umbilical cord bleeding	45/55 (85%)	0
Central nervous system bleeding	3/55 (10%)	4/100 (4%)
Hemarthrosis	30/55 (54%)	86/100 (86%)
Muscle hematoma	40/55 (72%)	93/100 (93%)
Gastrointestinal bleeding	0	10/100 (10%)
Urinary tract bleeding	0	12/100 (12%)
Epistaxis	40/55 (72%)	50/100 (50%)
Menorrhagia	14/20 (70%)	n.a.
Oral cavity bleeding	40/55 (72%)	55/100 (55%)
Postoperative bleeding	23/55 (40%)	36/100 (36%)
Thrombotic symptoms	2/55 (4%)	0

n.a. denotes not applicable

Results and discussion

Symptoms in patients with afibrinogenemia are shown in Table 1, which also shows the corresponding data in 100 patients with hemophilia A matched for age (range 4-71 years) and severity (factor VIII 1% or less). The most frequent and life-endangering hemorrhagic symptom was umbilical cord bleeding (85% of patients). Bleeding in the central nervous system was relatively rare (in 3 of 55 patients) at variance with other

reports (Fried & Kaufman, 1980).
Hemarthroses and hematomas were frequent (54% and 72%, respectively), but only 15 patients had clinical signs of musculoskeletal damage. While hematuria and gastrointestinal bleeding never occurred in this series, bleeding in other mucosal tracts such as epistaxis and menorrhagia were frequent (72% of all patients the former, 70% of women the latter). Circumcision and other surgical maneuvers were sometime accompanied by excessive oozing and impaired wound healing (40%).
Overall, it appears that in afibrinogenemia bleeding symptoms are qualitatively different and somewhat less severe than in hemophilia. For instance, a smaller proportion of patients had spontaneous joint and muscle bleeding, the most common and typical symptom in hemophilia (Table 1). Permanent damage to the musculoskeletal system and the resulting handicaps were definitely rarer. Hematuria, not unusual in hemophiliacs, was absent in this series. On the other hand, umbilical cord bleeding, never seen in hemophiliacs, was highly prevalent among afibrinogenemic patients. The relatively high frequency of mucosal-type symptoms such as nose bleeding and menorrhagia might be explained by the fact that in afibrinogenemia platelet fibrinogen is usually deficient (Roberts & Bingham, 1998).
There have been reports of decreased fertility and recurrent abortions in afibrinogenemic women (Goodwin, 1989). Recurrent abortions (more than two and consecutive) were seen in three of 18 married Iranian women. One had two miscarriages and never completed a pregnancy, another had four consecutive miscarriages and the third after two miscarriages eventually delivered a normal child after prophylactic treatment with cryoprecipitate twice weekly during the first six months of pregnancy. Therefore, afibrinogenemic women tend to have recurrent abortions.
Perhaps, as shown in mice made fibrinogen deficient by abrogating the a-chain gene (Suh et al, 1995), afibrinogenemia affects the implantation of the embryo.
Arterial or venous thromboses have been reported to occur spontaneously or after infusion of fibrinogen-containing preparations in afibrinogenemic patients (see Roberts & Bingham, 1998). In our series, a 5-year-old boy developed paraplegia as a consequence of thrombosis of the cerebral sagittal sinus. A 14-year-old girl developed ischemic gangrene of the right foot due to the thrombotic occlusion of the popliteal artery and the amputation of the toe was eventually necessary. Both thrombotic complications occurred at a young age and in the absence of fibrinogen infusion or circumstantial risk factors. The puzzling association of a severe coagulation defect such as afibrinogenemia and thrombosis found previously and in this series has as yet no definite explanation. It has been suggested that thrombotic events are related to thrombin-induced platelet aggregation in vivo due to poor neutralization of this enzyme, in turn due to lack of its adsorption on fibrin (Chafa et al, 1995).

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Symptoms of inherited factor V deficiency in 35 Iranian patients

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Summary

The type of bleeding symptoms have been evaluated in 35 Iranian patients with the inherited deficiency of factor V, with plasma levels between 1% and 10%. The most frequent symptoms included epistaxis and excessive bleeding after surgery.

Hemarthroses and muscle hematomas were less common, even in severely deficient patients. More severe symptoms such as gastrointestinal and central nervous system bleeding were rare. The severity of bleeding symptoms was only partially related to the degree of factor V deficiency in plasma. On the whole, human factor V deficiency is characterized by a moderately severe bleeding phenotype.

Key words: factor V deficiency, inherited coagulation disorders, bleeding symptoms

Introduction

In the most frequent congenital coagulation disorders such as hemophilia A and B, von Willebrand disease and factor XI deficiency, the pattern of the clinical manifestations of the bleeding tendency is well established. Other congenital coagulation defects are very rare (frequencies of less than 1 in one million in the general population; Girolami *et al*, 1985). Accordingly, information on the spectrum of bleeding symptoms is limited and scattered, being based upon reports of single cases or small series of patients. In countries where consanguineous marriages are frequent, genetic diseases transmitted with an autosomal recessive pattern of inheritance are relatively more prevalent. In Iran recessive coagulation disorders occur approximately ten times more frequently than in Western countries. We analyzed the clinical manifestations of 35 Iranian patients diagnosed with a congenital deficiency of factor V (Owren, 1947).

Patients and methods

The subjects comprised 25 males and 10 females, ages 3 to 65 years, belonging to 30 unrelated families. In 24 families patients were born from marriages between first-or second-degree cousins. To minimize bias and to evaluate critically the reliability of the clinical history as reported by patients, a specially tailored questionnaire (available on request) was delivered to the patients by one of us (M.L.). A descriptive threshold was used below which a reported bleeding symptom was not taken into consideration. Epistaxis was accepted when it was recurrent, from both nostrils and lasted longer than 10 minutes or required hospital admission. Menorrhagia was defined as menstrual periods of at least 6 days needing hormonal treatment or causing iron deficiency. Gastrointestinal bleeding, haematuria and intracranial bleeding had to be documented by hospital records. Hematomas and hemarthroses were classified when they caused the transient or permanent presence of clinical or radiological signs of joint or muscle impairment. Oral bleeding was classified as any episode lasting longer than 10 minutes, caused by tooth extractions or eruptions or by bites to lips, cheek and tongue. Surgical and post-partum bleeding was

considered any bleeding causing a delay in discharge from hospital or requiring blood transfusion. Bleeding symptoms occurring after surgery (including circumcision) were considered only when they occurred before diagnosis and patients were not treated prophylactically.

Plasma levels of factor V coagulant activity were measured with a prothrombin time-based manual assay using rabbit thromboplastin (Ortho Diagnostic System) and factor V deficient plasma obtained from a congenitally deficient patient with unmeasurable plasma levels (normal laboratory range: 71-125%). Factor VIII coagulant activity was also measured in all patients to exclude the combined deficiency of factor V and VIII, which is frequent in Iran (Peyvandi *et al*, 1998a). Patients were classified in three groups: severe (16 patients with factor V 1% or less), moderate (13 patients with 2-5% factor V) and mild (6 patients with factor V 6-10%).

Table 1 - **Prevalence of bleeding symptoms in congenital factor V (FV) deficiency**

Symptom	All patients (n=35)	FV 1% or less (n=16)	FV 2-5% (n=13)	FV 6-10 (n=6)
Epistaxis	20/35 (57%)	10/16	5/13	5/6
Menorrhagia	5/10 (50%)	3/4	2/6	0*
Gastrointestinal bleeding	2/35 (6%)	0/16	2/13	0/6
Hematuria	2/35 (6%)	1/16	1/13	0/6
Hemarthrosis	9/35 (26%)	5/16	2/13	2/6
Muscle hematoma	10/35 (29%)	7/16	2/13	1/6
Central nervous system bleeding	2/35 (6%)	2/16	0/13	0/6
Umbilical cord bleeding	1/35 (3%)	1/16	0/13	0/6
Post-operative and post-partum bleeding	15/35 (43%)	7/16	4/13	4/6
Oral cavity bleeding	20/35 (57%)	9/16	7/13	4/6

** There was no woman of child-bearing age in this category of factor V levels*

Results

Table 1 summarizes the number and prevalence of each bleeding symptom in patients divided into the three groups of plasma factor V levels.

Soft tissue bleeding. The most frequent symptom was bleeding in the oral cavity (57% of all cases), which occurred after bites to the tongue or lips or following the extraction of permanent teeth. This symptom occurred with similar frequency in the three categories of factor deficiency, even though in severely deficient patients bleeding tended to be more severe. Excessive bleeding was also found after surgery (43%), most often after circumcision, whatever the degree of factor deficiency. Hemarthroses and hematomas occurred in about one-fourth of the patients, including those with milder deficiency. In no patient was there any musculo-skeletal damage of clinical significance, but early signs of arthropathy were seen on radiological or ultrasound examination in all patients who had hemarthroses. Subdural and intracerebral bleeding

occurred in two patients with severe deficiency and one child had prolonged bleeding when the umbilical stump fell.

Mucosal bleeding. Epistaxis was relatively frequent (57%) and occurred even in patients with mild deficiency (5 of 6 cases). Menorrhagia occurred in 5 of the 10 women of child-bearing age, all had severe or moderate factor deficiency. Other symptoms such as gastrointestinal and urinary tract bleeding were rare, with no triggering lesion identified in the 4 patients who had these symptoms.

Discussion

Since only 150 factor V deficient patients are known so far (Girolami *et al*, 1985), this large series contributes substantially to our knowledge of the spectrum of the clinical manifestations of this disorder. In most patients bleeding symptoms first developed during the first six years of life, but only one bled from the umbilical stump. There was no instance of cephalohematoma. Epistaxis was frequent, even in patients with moderate and mild deficiency. In contrast, other symptoms of mucosal bleeding such as melena and hematuria were relative rare. We have previously found a surprisingly high frequency of epistaxis in the inherited deficiencies of factor VII and X (Peyvandi *et al*, 1997 and 1998b). It remains to be explained why epistaxis is more frequent in recessive coagulation disorders than in hemophilia A and B. Postoperative and oral cavity bleeding was relatively common, but not predictable, as these symptoms also occurred in patients with 5 to 10% plasma levels. Variations in the platelet levels of factor V and in the skin bleeding time, not measured in our patients, may explain this discrepancy (Bredeerveld *et al*, 1975). Hemarthroses and hematomas occurred in 26 and 29% of the patients, but were not the most frequent and severe symptoms as in classic hemophilia. On the whole, in factor V deficient patients hemorrhagic manifestations are not as severe or frequent as in hemophiliacs with corresponding degrees of coagulation factor deficiency. This moderately severe phenotype sharply contrasts with the dramatic early-lethal bleeding phenotype found in knock-out mice lacking factor V (Cui *et al*, 1996). Perhaps the genetic lesions of the Iranian patients are such that traces of factor V can be produced, rendering the phenotype less severe.

Factor V is the only inherited bleeding disorder for which no concentrate is available.

Fresh-frozen plasma is the mainstay of treatment in Iran. In our experience, single daily dosages of 15-20 ml/kg are usually adequate to control most spontaneous hemorrhages in soft tissues and mucosal tracts. A frequent symptom such as epistaxis can be usually controlled by local measures and antifibrinolytic drugs, so that replacement therapy is rarely necessary. In a few surgical cases, prolonged treatments with large amounts of fresh-frozen plasma caused volume overload. Therefore, a factor V concentrate is warranted.

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Clinical manifestations in 28 Italian and Iranian patients with severe factor VII deficiency

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Summary

There has been wide variation in the reported haemorrhagic manifestations of factor VII deficiency. We examined type and frequency of clinical manifestations in 28 Iranian and Italian patients with severe deficiency (factor VII coagulant activity 2% or less). The most frequent symptoms were epistaxis and menorrhagia, whereas soft tissue bleeding such as haemarthrosis and muscle haematoma was less frequent. Only 5 of 9 patients who underwent surgery without factor VII replacement therapy had postoperative bleeding severe enough to require blood transfusion. No thrombotic manifestation occurred. A factor VII functional assay based on the use of human thromboplastin was a better predictor of the bleeding tendency of these patients than a rabbit thromboplastin-based functional assay or immunoassay. On the whole, this study shows that in severe factor VII deficiency bleeding in mucosal tracts is not uncommon. Surgery can sometimes be performed without replacement therapy and without haemorrhagic complications.

Keywords: factor VII, factor VII deficiency, congenital bleeding disorders.

Introduction

Congenital deficiency of factor VII, first described in 1951 by Alexander *et al.* (1), is an autosomal recessive disorder of blood coagulation.

According to textbooks, prevalence in the general population is 1:500 000, accounting for approximately 0.5% of all congenital coagulation disorders (2). The Italian Registry of Congenital Coagulation Disorders lists 57 patients with severe or moderate deficiency of factor VII (less than 5%), so that the prevalence of the defect in Italy is 1:950 000, close to that quoted in textbooks. From reports in the literature, in general single cases or small series, it appears that the type and frequency of clinical manifestations of this rare disorder are quite varied (3-6). In the framework of a collaboration between the haemophilia centres of Milan in Italy and of Tehran in the Islamic Republic of Iran (where factor VII deficiency is relatively frequent because consanguineous marriages are customary among Muslims), we chose to reassess the issue of variation in clinical manifestations of factor VII deficiency. Twenty-eight patients with severe deficiency, the largest series ever reported in a single study, have been examined by the same physician. Plasma levels of factor VII coagulant activity (VII:C) and factor VII antigen (VII:Ag) were also measured in the attempt to correlate phenotypes with the type and frequencv of clinical manifestations.

Patients and methods

Iranian patients. This group consisted of 18 patients (six women, 12 men, age range 8-5 years) belonging to 14 families selected solely on factor VII levels (see below) and availability to attend the Tehran He-mophilia Centre for interview, clinical examination and blood sampling. On the basis of the historical values of plasma VIII:C levels as obtained using a rabbit thromboplastin-based functional assay, patients with less

than 2% VII:C were selected for this study. In 12 of the 14 families factor-VII-deficient patients were born from marriages berween flrst-degree (n = 10) or second-degree (n = 2) cousins.

Italian patients. This group consisted of 10 patients (seven women and three men, age range 6-53 years), belonging to seven different families. They were selected with the same criteria of the Iranian patients, including cut-off VII:C levels. In none of the families was there consanguineity.

Collection of clinical and laboratory data

Patients from both groups attended the haemoplilia centres of Milan and Tehran, where the same physician (F.P.) examined them, constructed family pedigrees and collected the medical history, with specific questions on the occurrence of bleeding and thrombotic symptoms. A structrured questionnaire was used for this purpose. For symptoms such as bruising, haematoma and haemarthrosis, questions were focused on the presence or no of a traumatic cause, on severity, frequency and localization; for epixtaxis, on frequency, occurrence from both nostrils and need for local and systemic therapy; for menorrhagia, on duration of the menstrual period and on the number of absorbents used; for melena, haematuria and CNS bleeding, on clinical manifestations and severity; for postsurgical and postextraction bleeding, on duration, recurrence, wound haematoma formation and need for transfusional or haemostatic therapy.

Plasma preparation and storage. Blood samples were collected in siliconized vacuum tubes containing 0.129 M buffered trisodium citrate and plasma was separated by centrifugation at 20°C, 2000g for 10 min. Platelet-poor plasma was subdivided in aliquots, put in plastic tubes, snap-frozen in a dry ice - methanol-acetone mixture and stored in the deep-freezer at -70°C. The Iranian samples were transported frozen from Tehran to Milan in containers with dry ice.

VII:C assays. VII:C was measured using a previously described functional assay (7). Factor-VII-deficient plasma was purchased from Dade International (Miami, FL, USA). Three types of thromboplastins were used to obtain three sets of VII:C values: rabbit thromboplastin (OBT, Ortho Diagnostic System, Raritan, NJ, USA), human recombinant thromboplastin (Recombiplastin, Ortho Diagnostic System) and bovine thromboplastin (Instrumentation Laboratory, Milan, Italy). VII:C assay were carried out manually and VII:C levels were calculated using as standard frozen plasma pooled from 40 normal individuals (20 men and 20 women not pregnant and not taking oral contraceptives). Standard plasma was given an arbitrary value of 100% VII:C and patient values were calculated accordingly.

VII:Ag assay. VII:Ag was measured using a previously described enzyme immunoassay (8). This method is based on the use of murine monoclonal antibodies against human factor VII. VII:Ag levels were calculated using as standard the same pooled plasma used for VII:C assays and the same method of result expression.

Results

Since there was no substantial difference between the Iranian and Italian patients for demographic features and factor VII levels, the results obtained in the two groups are presented together (Table 1).

Table 1 shows the VII:C and VII:Ag levels obtained for each thromboplastin. In the majority of patients (n = 23) there was an excellent correspondence between the very low levels of VII:C (2% or less) obtained with each thromboplastin. On the other hand, in four Iranian patients belonging to the same sibship and in an additional unrelated Iranian patient, unmeasurable VII:C levels with the rabbit thromboplastin-based assay contrasted with low levels obtained with human thromboplastin (17-37%) and normal levels with bovine thromboplastin (70-130%). Table 1 also shows that these patients had normal to very high levels of VII:Ag (106-240%). In the remaining 23 patients, VII:Ag levels ranged from unmeasurable (less than 1%, three cases) to low (1-50%, nine cases) or normal (51-150, 11 cases).

Table 1 also gives the bleeding symptoms observed lifelong in each patient and Table 2 summarizes the overall prevalence for each symptom. For some symptoms the number of patients considered is smaller than the total number of 28, because for menorrhagia only women in the fertile age were considered and for dental extractions and surgery only those who underwent procedures without replacement therapy. The most frequent bleeding symptoms were epistaxis and menorrhagia. Other haemorrhages in mucosal tracts, such as haematuria and melena, occurred only in three and four patients. Soft-tissue bleeding was relatively uncommon, as haemarthrosis was reported in six patients, muscle haematomas in three and easy bruising in nine. CNS bleeding had occurred in five patients (four cases of intracerebral bleeding and one case of subarachnoidal bleeding). In patietits who had undergone dental extractions, bleeding was reported to be excessive and prolonged in 10/15 cases. Major surgery or circumcision had been performed in nine patients: five of them had excessive bleeding, which required blood transfusion in four (Table 3). No thrombotic episode had occurred.

There was no relationship between the patient phenotype, expressed by VII:C and VII:Ag levels, and the frequency of bleeding. Exceptions were the Iranian patients with a clear discrepancy between unmeasurable VII:C levels with rabbit thromboplastin and higher levels with human or bovine thromboplastins (Table 1). Two of them had no bleeding symptom, in spite of the fact that during their life span (ages 30 and 55 years) they had undergone multiple dental extractions or surgical procedures. The remaining three patients had relatively mild symptoms (menorrhagia and postextraction bleeding).

Discussion

The epitome of congenital coagulation defects is haemophilia. In patients with severe factor VIII or IX deficiency symptoms are quite typical, with a prevalence of soft-tissue haemorthages (haemarthrosis, muscle haematomas) and a relative paucity of mucosal tract

Tab. 1 - **Demographic features, haemorrhagic symptoms and plasma levels of factor VII coagulant activity (VII:C) with different thromboplastin and of factor VII (VIIAg) in Italian and Iranian patients**

Family	Patient	Sex/ age	Country	Easy bruising	Haemarthrosis	Haematoma	Epistaxis	Melena	Haematuria	Menorrhagia
1	I	M/28	Italy				•			
2	I	F/53	Italy	•	•		•		•	•
	II	M/53	Italy		•	•				
3	I	F/38	Italy	•	•					•
4	I	F/39	Italy	•			•			•
	II	F/34	Italy				•			
	III	F/33	Italy				•			
5	I	M/6	Italy	•			•			
6	I	F/13	Italy				•			
7	I	F/7	Italy					•		
8	I	F/51	Iran	•						
	II	F/45	Iran							•
	III	M/55	Iran							
	IV	F/30								
9	I	F/21	Iran							•
10	I	F/14	Iran			•				
11	I	M/9	Iran	•			•			
12	I	M/27	Iran				•			
13	I	M/15	Iran				•	•		
14	I	M/17	Iran		•	•				
15	I	M/8	Iran				•			
16	I	F/30	Iran	•			•			•
	I	M/12	Iran				•			
17	I	M/8	Iran		•		•		•	
18	I	M/24	Iran				•			
19	I	M/15	Iran				•	•		
20	I	M/15	Iran	•	•		•	•		
21	I	M/18	Iran	•			•		•	

haemorrhages such as epistaxis and melena (2). The only exception to this pattern is haematuria, not unusual in haemophilia. When dental extractions and surgical procedures are carried out without replacement therapy, prolonged and life-threa-tening bleeding occurs almost invariably. Since Factor VII deficiency is a congenital defect of blood coagulation like factor VIII and IX deficiencies, one would expect similar clinical symptoms.

CNS bleeding	Post surgical bleeding	Post extraction bleeding	VII:C rabbit	VII:C human	VII:C bovine	VII:Ag
		•	<1	<1	<1	3
•	•	•	<1	<1	<1	33
			<1	<1	<1	42
			<1	<1	<1	<1
•	•	•	1	<1	<1	54
			2	1	<1	43
			2	1	<1	55
•			<1	<1	<1	<1
			<1	<1	<1	5
•			<1	<1	<1	2
		•	<1	37	130	240
			<1	27	90	224
			<1	24	100	156
			<1	17	70	106
		•	<1	22	80	130
			<1	<1	<1	77
			<1	<1	<1	78
		•	<1	<1	<1	104
•			<1	<1	<1	132
			<1	<1	<1	135
			<1	<1	<1	<1
		•	<1	<1	<1	133
	•		<1	<1	<1	80
	•	•	<1	<1	<1	6
		•	<1	<1	<1	129
			<1	<1	<1	101
			<1	<1	<1	4
			<1	<1	<1	1

Our patients and those of Mariani & Mazzucconi (6), which make the two largest series of patients with severe or moderate factor VII deficiency reported (28 and 24 cases, respectively), are not totally consistent with this prediction. In both studies a most prevalent symptom was epistaxis (Table 2). The reasons for the frequency of this symptom, more typical of platelet than of coagulation defects, is not clear at the moment. Even though the skin bleeding time was not measured because this test is not part of our screening of coagula-tion defects, others have found prolongations of the bleeding time in patients with factor VII deficiency (9). These observations, and the relative frequency of mucosal bleeding in the presence of normal platelet count, warrant a study of platelet function in factor-VII-deficient patients.

Tab. 2 - **Prevalence of each bleeding symptom in patien with factor VII deficiency.**

Symptoms	No. with symptoms/ no. considered	Prevalence	Prevalence in Mariani's series (ref.6)
Easy bruising	9/28	32%	29%
Haemarthrosis	6/28	21%	66%
Haematoma	3/25	12%	45%
Epistaxis	18/28	64%	62%
Melena	4/28	14%	16%
Haematuria	3/28	10%	29%
Menorrhagia	6/10	60%	90%
CNS bleeding	5/28	17%	n.r
Postsurgical bleeding	5/9	55%	n.r.
Postextraction bleeding	10/15	66%	25%

n.r.=data not reported.

Our patients, while consistent with those reported by Mariani & Mazzucconi (6) for the high frequency of mucosal bleeding, do not confirm their findings of a high frequency of haemarthrosis (16 of 24 patients) (Table 2).

Tab. 3 - **Surgery and bleeding in nine patients with factor VII deficiency**

Patient code	Type of operation	Bleeding complications
2/I	Tonsillectomy	yes*
	Appendectomy	yes*
4/I	Tonsillectomy	yes*
4/II	Rhinoplasty	no
4/III	Appendectomy	no
8/III	Circumcision	no
12/I	Circumcision	no
16/I	Circumcision	yes
17/II	Thoracotomy	yes*
19/I	Circumcision	yes*

**Required blood transfusion.*

Even though a few of our patients (6/28) had arthropathy as a consequence of recurring joint bleeding, the majority of them never had haemarthrosis in spite of the fact that, unlike those in the study by Mariani & Mazzuconi, all had severe factor VII deficiency (2% or less). It would appear therefore that haemarthrosis is not the most typical clinical manifestations of factor VII deficiency. In their review of 75 cases reported in the literature, Ragni *et al.* (4) pointed out a relatively high prevalence of CNS bleeding (16%). Our data are consistent with their observation, with five of 28 cases (17%). Goodriough *et al.* (10) were the first to describe factor-VII-deficient patients with a positive personal history for thrombosis, with clinical manifestations such as myocardial infarction, pulmonary embolism, superficial thrombophlebitis, angi-na pectoris and caval vein thrombosis. In our patients, some of whom had reached a relatively advanced age and hence have been exposed to a relatively high risk, no thrombotic episode was reported. In this and in Mariani's series, the existence of an association between factor VII deficiency and thrombosis is not apparent. This is not surprising because it is not biologically plausible that a se-vere coagulation defect determines a prothrombotic state.

The large heterogeneity of laboratory findings observed in our patients with factor VII deficiency has been observed previously. Goodnight *et al.* (11) were the first to document such heterogeneity in patients who had a discrepancy between low levels of VII:C and higher levels of VII:Ag. Subsequently, the existence of immunological variants of factor VII deficiency has been confirmed by several investigators. Triplert *et al.* (5), studying 26 patients with low VII:C levels and varied VII:Ag levels, observed that VII:C values obtained with human thromboplastin-based assays correlated better with clinical symptoms than those obtained with rabbit thromboplastin. This is confirmed in five members of two Iranian kindreds characterized by higher VII:C levels with human and bovine thromboplastin than with rabbit thromboplastin. This functional factor VII variant (called Padua 1) was first described by Girolami *et al.* (12) in an Italian patient and subsequently found in additional Italian, French and Japanese patients (13-15). Both in the four previously reported cases and in the five cases identified by us bleeding symptoms were mild or totally

absent. The quasi normal reactivity of factor VII Padua with homologous (human) tissue factor is likely to explain this situation.

On the whole, this study demonstrates that the symptoms of patients with factor VII deficiency are more similar to those of a combined vascular coagulation defect such as von Willebrand disease than to those of a typical coagulation defect such as haemophilia A. The reasons for this behaviour are unknown at the moment and warrant the study of platelet function in these patients. This study confirms the occurrence of a very high phenotypic heterogeneity in factor VII deficiency, consistent with the heterogeneity of the molecular defects found in these patients at the DNA level (16). In the screening of coagulation defects, VII:C assays with human thromboplastin should be preferred, because rabbit thromboplastin-based assays may give very abnormal results in individuals with a small bleeding risk.

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Bleeding symptoms in 27 Iranian patients with the combined deficiency of factor V and factor VIII

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Summary

Inherited deficiency of factors V and VIII is the most frequent combined coagulation defect. The cases reported so far are mostly single cases or small series from different centers, making it difficult to evaluate the overall pattern of clinical manifestations of the combined defect. We examined at a single institution 27 Iranian patients.

Mucocutaneous and post-surgical bleeding were the most frequent clinical manifestations. The presence of two defects did not make the severity of bleeding greater than that expected in patients with single coagulation defects of similar degrees.

Key words: factor V, factor VIII, combined factor V and factor VIII deficiency

Introduction

The inherited combined deficiency of factor V and factor VIII, first described by Oeri *et al* (1954), has been subsequently reported in at least 62 families (106 cases) according to our current review of the literature. A single genetic defect, transmitted with an autosomal recessive pattern of inheritance, is thought to be responsible for the combined deficiency. Recently the gene has been localized to chromosome 18q (Nichols *et al*, 1997a; Neerman Arbez *et al*, 1997) and the protein product of the gene is a component of the endoplasmic reticulum-Golgi intermediate compartment, previously termed ERGIC 53 (Nichols *et al*, 1997b).

The homozygous phenotype of the combined deficiency is usually characterized by plasma levels of factor V and VIII coagulant activities ranging between 4 and 30%, with correspondingly low levels of immunoreactive proteins (Soff & Levin, 1981; Giddings *et al*, 1982). Whereas in congenital defects of single coagulation factors the pattern of bleeding symptoms and their relationship to plasma factors levels are quite well established, relatively little information is available for combined defects, of which factor V and VIII deficiency is the archetype. From published reports, in general single cases or small series, it would appear that in homozygotes the type and severity of clinical manifestations are variable, whereas heterozygotes, who have borderline or normal levels of factors V and VIII, are usually asymptomatic (Soff & Levin, 1981; Seligsohn *et al*, 1982). In Iran the combined deficiency of factor V and VIII is relatively frequent (like other autosomal recessive coagulation disorders; see Shoa'i *et al*, 1977; Peyvandi *et al*, 1997), because consanguineous marriages are frequent among Muslims. We chose to reassess the pattern of the clinical manifestations in a large series of Iranian patients.

Patients and methods

The combined deficiency was diagnosed when factor V coagulant activity was below 40 per cent and factor VIII coagulant activity below 45 per cent.

These cut-off values are the lower limits of the normal factor distribution at the Tehran Hemophilia Centre. The patients studied were 27 (12 women, 15 men, age range 6-48 years). In 14 of the 16 families patients were born from marriages between first-degree

(n=12) or second-degree (n=2) cousins. Patients were selected solely on factor V and VIII levels (see above) and availability to attend the Hemophilia Centre for interview, clinical examination and blood sampling. Three physicians (E.G.D.T., F.P and M.A.) examined them, constructed family pedigrees and collected the medical history, with questions tailored to evaluate the significance of the bleeding symptoms reported. For hematoma and hemarthrosis, questions were focused on the presence or not of a traumatic cause, on localization and frequency and on the presence or not of functional and radiological joint and muscle damage; for epistaxis, on frequency (at least two episodes without a history of trauma), and on the need for compression or medical treatment; for menorrhagia, on duration of the menstrual period (6 days or more), number of absorbent pads used daily (10 or more) and occurrence of iron-deficiency anemia; for melena, hematuria and intracranial bleeding, on the need for hospital treatment; for circumcision, dental extractions and other surgical procedures, on duration of bleeding, recurrence, wound hematoma formation and the need for hospital admission or transfusion therapy. Bruising was not considered, because it was difficult to evaluate the reliability of this symptom as reported by patients.

Factor assays. Factor V coagulant activity was measured with a prothrombin-time-based assay, using factor V deficient plasma (less than 1%) produced by ageing at 37° C human plasma collected in EDTA and a rabbit thromboplastin (OBT, Ortho Diagnostic System, Raritan, NJ). Factor V levels were calculated using as standard frozen plasma pooled from 40 healthy men and 2 women. Standard plasma was given an arbitrary value of 100% and patient values were calculated accordingly. Factor VIII coagulant activity was measured with an APTT-based assay, using as substrate citrated plasma from a patient with severe hemophilia A (factor VIII levels less than 1 %). Factor VIII levels were calculated as for factor V.

Results

Table 1 shows that the factor V level ranged from 2 to 21% (median: 11%), factor VIII from 2 to 22% (median: 13%). Eleven patients had levels of both factors of 10% or lower, 8 had both factors above 10%, whereas the remaining 8 had one factor above and the other below the 10% limit.

Table 1 also gives the bleeding symptoms that had occurred lifelong in each patient. For some symptoms the number of patients is less than 27, because for menorrhagia only the 12 women in the fertile age were considered and for circumcision only the 11 boys who underwent the procedure without replacement therapy were taken into account. The most frequent spontaneous bleeding symptoms were of the mucosal type, epistaxis (21/27) and menorrhagia (7/12).

Gastrointestinal bleeding occurred in 2 patients, hematuria in none. Soft tissue bleeding was less frequent, as spontaneous hemarthrosis was reported in 7 patients and muscle hematoma in two (Table 1). In two patients (IV-2, factor V 5% and factor VIII

Tab. 1 - **Bleeding symptoms in 27 patients with combined factor V and factor VIII deficiency**

Family	Patient	Sex	Age (yr)	Factor V activity (%)	Factor VIII activity (%)	Epistaxis	Menorrhagia	Gastrointestinal bleeding
I	1	M	25	8	6	•		
	2	F	10	11	9	•		
	3	F	17	7	14	•	•	
II	1	F	26	8	7	•	•	
III	1	F	21	20	15			
IV	1	F	33	21	18	•		
	2	M	37	5	22	•		
V	1	F	17	7	9	•	•	
VI	1	F	15	15	14		•	
	2	M	14	14	13	•		
VII	1	M	43	13	8	•		•
VIII	1	M	24	18	21	•		
	2	M	34	9	8	•		
	3	M	12	5	6			
IX	1	M	29	18	8	•		
X	1	M	12	19	16	•		
	2	F	20	12	15	•	•	
XI	1	M	43	18	17	•		
XII	1	M	22	2	2	•		
XIII	1	F	14	5	6	•	•	
XIV	1	F	35	10	13	•		
	2	M	45	10	6			•
	3	F	30	10	6			
	4	F	34	10	21	•	•	
XV	1	M	48	9	14			
XVI	1	M	6	10	8	•		
	2	M	32	7	5	•		

•= *presence of the symptom* *N= no excessive bleeding after the procedure or event*

22%; and XII-1, factor V 2% and factor VIII 2%) spontaneous joint and muscle bleeding had led to actual severe functional impairment and radiological damage in the hip and knee joints, with a large femural pseudotumor in patient XII-1. Intracranial bleeding had occurred in one patient, with no obvious traumatic cause. Among the 17 patients who had dental extractions without replacement therapy, bleeding was reported to be excessive and prolonged in 14 cases (one case required blood transfusion). Eight of the 12 boys

Haemarthrosis	Haematoma	CNS bleeding	Post-circumcision bleeding	Post-extraction bleeding	Post-operative bleeding	Post-partum bleeding
			•			
				N		
•		•		•		
				•	•	
				N		•
•			•	•		
				•	•	
•				•		
•			N	•		
			•	•		
			N	N		
•				•	•	
			•	•		
			N	•		
			N	•		
•				•		
				•		
•	•		•			
						N
			•			
				•		•
						•
	•		•		N	
			•			

who underwent circumcision without replacement therapy had excessive bleeding that required blood transfusion in one. In four patients who had major surgery without replacement therapy, three had excessive bleeding but no transfusion. After parturition, 3 of 4 women had excessive bleeding, but no transfusion. There was no strong relationship between the phenotypic severity, expressed by the combinations of low factor V and VIII levels, and the frequency of bleeding: the 11 patients with levels of both fac-

tors below 11% had 30 different bleeding symptoms lifelong, the 8 patients with levels above 11%, 19 symptoms.

Discussion

Until now, the largest series of patients with combined factor V and VIII deficiency examined at a single institution were 14 homozygotes from 8 unrelated Oriental Jewish families reported by Seligsohn *et al* (1982), who estimated a frequency of homozygous disease of 1:100.000 in Oriental Jews. Our series of 27 patients, none of whom originated from the community of Iranian Jews, are the largest series ever reported. In Iran, a total of 64 patients are currently known to have the combined deficiency of factor V and VIII, with symptoms severe enough to postulate that they are homozygotes. Since the population of the country is 60 millions, a rough estimate of the frequency of the combined deficiency is 1:100.000, similar to that found in Oriental Jews. Consanguineous marriages and the low fatality rate of the disease, even with little available treatment, are likely to explain the relatively high frequency of this recessive bleeding disorder.

The main goal of this retrospective study was to establish the bleeding manifestations typical of the homozygous state. Patients were examined at a single institution, trying as far as possible to use homogeneous and objective criteria to ascertain the significance of the bleeding symptoms reported. Patients were phenotypicall quite homogeneous, with median levels of factor V and VIII around 10%, typical of the homozygous state for the combined deficiency (Soff & Levin, 1981; Giddings *et al*, 1982; Seligsohn *et al*, 1982). Preliminary data indicate mutational heterogeneity at the ERGIC-53 locus in the Iranian families described here in (unpublished observation). The most commonly noted clinical manifestations were epistaxis, menorrhagia and excessive bleeding after surgical trauma and delivery, in agreement with Seligsohn *et al* (1982). Surprisingly, Seligsohn *et al* (1982) found no case of excessive bleeding after circumcision, whereas two thirds (8/12) of our male patients bled excessively when the procedure was carried out without replacement therapy. Whereas circumcision is performed at birth in Jews, it is usually performed at 5-7 years of age in Muslims. Perhaps the trauma of the operation is less at birth than during childhood. Spontaneous soft tissue bleeding was relatively infrequent, but two patients had signs of severe arthropathy.

On the whole, the type and severity of symptoms were similar to those that occur in patients with single deficiencies of factor V and factor VIII, with a predominance of mucocutaneous bleeding and post-operative bleeding. There is no clear evidence that the severity of bleeding is heightened by the concomitant presence of two coagulation defects.

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Congenital factor X deficiency: spectrum of bleeding symptoms in 32 Iranian patients

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Summary

The spectrum of the clinical manifestations of congenital factor X deficiency was studied in 32 patients from Iran. The most frequent symptom was epistaxis, which occurred in 72% of patients, with all degrees of deficiency. Other mucosal hemorrhages such as hematuria and gastrointestinal bleeding were less frequent and occurred mainly in patients with unmeasurable factor X. Menorrhagia occurred in half of the women in the reproductive age. Soft tissue bleeding occurred in two-thirds of the patients, and was characterized by spontaneous hematomas and hemarthroses that in 5 patients led to severe arthropathies. Bleeding from the umbilical stump was an unexpected finding in 9 patients. This study demonstrated that bleeding tendency of factor X deficiency is severe and correlates with factor levels.

Key words: Factor X, Factor X deficiency

Introduction

Congenital deficiency of coagulation factor X, first described over 30 years ago (Telfer *et al*, 1956; Hougie *et al*, 1957), is usually diagnosed by a concomitant prolongation of the prothrombin time and activated partial thromboplastin time in patients who bleed. The disease is rare (only approximately 50 families have been reported) and genetic transmission is autosomal recessive (usually, only homozygotes are symptomatic) (Perry, 1997). Until now only single cases or small series of patients have been reported, so that the spectrum of the clinical manifestations of the disease is not well established. We report a large series of patients with factor X deficiency diagnosed and followed-up at the National Hemophilia Centre in Tehran. The study was undertaken because of the high frequency of factor X deficiency in Iran, a country where consanguineous marriages are frequent.

Patients and methods

Thirty-two patients with factor X deficiency (23 males, 9 females, age 5 to 72 years) from 25 unrelated Iranian families were studied. In 23 of the families, patients were born from marriages between first-or second- degree cousins. All patients were diagnosed and regularly followed up at the National Hemophilia Centre in Tehran, where detailed records of clinical events occurring in hospital or at home were kept. For the purpose of this study, patients were selected solely on factor X activity levels (see below) and their ability to attend the Hemophilia Centre for interview, physical examination and blood collection. The registry of congenital coagulation disorders kept at the National Hemophilia Centre lists 50 patients with factor X deficiency in the whole country, with a prevalence of the disease of 1 in 1.200.000 in the general population. Hence, the present cohort of 32 patients appears to be representative of the whole population. On the basis of the plasma levels of factor X coagulant activity (FX:c), measured with a prothrombin-time-based assay using rabbit thromboplastin and factor X deficient plasma (normal range: 65-120%), patients were classified in three groups: severe (18 patients

Table 1 - **Prevalence of bleeding symptoms in congenital factor X deficiency**

Symptom	All patients (n=32)	FX:c<1% (n=18)	FX:c 1-5% (n=9)	FX:c 6-10% (n=5)
Epistaxis	23/32 (72%)	11/18 (61%)	8/9 (88%)	4/5 (80%)
Menorrhagia*	4/8 (50%)	3/5	1/1	0/2
GI bleeding	12/32 (38%)	12/18 (66%)	0/9	0.5
Haematura	8/32 (25%)	7/18 (39%)	1/9	0/5
Haemarthrosis	22/32 (69%)	14/18 (77%)	7/9 (77%)	1/5
Haematoma	21/32 (66%)	14/18 (77%)	6/9 (66%)	1/5
CNS bleeding	3/32 (9%)	2/18 (11%)	1/9 (11%)	0/5
Umbilical cord bleeding	9/32 (28%)	7/18 (39%)	1/9 (11%)	1/5

** Eight women of reproductive age*

with less than 1% FX:c), moderate (9 patients with FX:c from 1 to 5%) and mild (5 patients with FX:c from 6 to 10%).

Collection of clinical data. All consultations were performed by the same physician (M.L.), who examined the patients, constructed family pedigrees and collected medical histories, with questions tailored to evaluate the significance of the bleeding symptoms reported. For hematoma and hemarthroses, questions and physical examination were focused on the presence or not of a traumatic cause, on localization and frequency and on the presence or not of functional and radiological joint and muscle damage; for epistaxes, on the frequency (at least three episodes from both nostrils without a history of trauma), and on the need for local compression or medical treatment; for menorrhagia, on the duration of the menstrual period (6 days or more), number of absorbent pads used daily (10 or more) or the occurrence of iron deficiency with no other source of bleeding; for gastrointestinal bleeding, hematuria and intracranial bleeding, on the need for hospital treatment.

Bleeding symptoms after dental extraction and minor/major surgery (including circumcision) were not included in the evaluation because we realized that most patients had been diagnosed early in life because of bleeding episodes. Therefore, they had usually received prophylactic treatment with fresh-frozen plasma or prothrombin complex concentrates before any surgical procedure. Bruising was not included, because it was difficult to evaluate the significance and reliability of this symptom as reported by patients.

Results

Table 1 summarizes the prevalence of each symptom in patients divided according to FX:c in plasma. Only one patient (FX:c 6%) had none of the bleeding symptoms evaluated in this study. Diagnosis was made because he bled excessively after a war wound.

Mucosal tract bleeding. The most frequent

spontaneous bleeding symptom was epistaxis, which occurred in 72% (23/32) of patients, in all severity groups. Eight of 9 women were in the reproductive age; menorrhagia occurred in four of them (50%). Gastrointestinal bleeding that led to anemia and hospital admittance occurred in 38% of all patients. None of these patients had lesions in the gastrointestinal tract that could explain the bleeding. Similarly there was no obvious cause for macroscopic hematuria, which occurred in 25% of patients. All except one of these patients have severe FX:c deficiency.

Soft tissue bleeding. Spontaneous hematomas and recurrent hemarthroses occurred in 66% and 69% of patients, mainly but not exclusively in those with severe and moderate deficiency. In 5 patients recurrent hemarthroses led to the development of a significant impairment of joint function. Intracerebral bleeding occurred in two patients with FX:c levels of<1% and in one with 3%. In them, bleeding occurred spontaneously and all patients eventually recovered with no residual neurological lesion. An unexpected finding in 9/23 patients (28%) was bleeding from the umbilical stump, which occurred in seven case with a severe deficiency and two moderate or mild deficiency.

Discussion

Factor X plays a pivotal role in blood coagulation, being at the intersection between the tissue factor and contact phase pathways of thrombin formation. Hence, patients with factor X deficiency might be expected to have particularly severe bleeding symptoms. The present study has attempted to address this by examining a large series of patients regularly followed-up at a single institution. Aware of the limits of a retrospective analysis of self-reported bleeding symptoms, we have attempted to minimize bias by using as much as possible hospital records and stringent criteria, such as the occurrence of anemia and the need for hospital treatment, before assigning bleeding symptoms to each patient.

The occurrence and frequency of bleeding symptoms correlated with the degree of factor X deficiency. An exception was epistaxis, that occurred in patients with all degrees of deficiency. On the other hand, other bleeding manifestations in mucosal tracts, such as hematuria and gastrointestinal bleeding, occurred almost exclusively in patients with unmeasurable factor X levels and no obvious anatomical lesion. The types of bleeding manifestations were similar to those seen in patients with factor VIII and IX deficiency (hemophilia A and B), with the exception of epistaxes and umbilical cord bleeding. Epistaxes were the most frequently reported symptom, an observation previously made in a series of 28 patients with severe factor VII deficiency studied with the same criteria (Peyvandi *et al*, 1997). Why this mucosal bleeding symptom, relatively unusual in patients with coagulation defects such as factor VIII and IX deficiencies, is so prevalent in factor VII and X deficiency is unclear. Bleeding from the umbilical stump is consi-

dered peculiar of factor XIII deficiency. In factor XIII deficiency, this symptom is usually delayed, because clot formation occurs normally at birth but stabilization of fibrin is impaired. In our series of factor X deficient patients, bleeding occurred between 7 and 10 days after birth, at the time of detachment of the stump, and 6 of 9 patients with this symptom had anemia severe enough to require blood transfusion.

In conclusion, this study of a large series of patients from a single institution indicates that the bleeding tendency of factor X deficiency may be severe and occurs early in life in patients with unmeasurable factor X levels. Mucosal and soft-tissue bleeding symptoms are frequent and umbilical cord bleeding, often considered indicative of factor XIII deficiency, may be one of the earlier manifestations.

Acknowledgments.

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chapter 3

Molecular studies

Molecular characterization and three-dimensional structural analysis of mutation in 21 unrelated families with inherited factor VII deficiency
Thromb Haemost 84: 205-7,2 000

Homozygous 2bp deletion in the human factor VII gene: a non-lethal mutation that is associated with a complete absence of circulating factor VII
Thromb Haemost, 84: 635-637, 2000

Characterization of two naturally occurring mutations in the second epidermal growth factor like domain of factor VII
Blood 93: 1237-1244, 1999

Abnormal secretion and function of recombinant human factor VII as the result of modification to a calcium binding site caused by a 15 base pair insertion in the factor VII gene
Blood, accepted for publication

Molecular analysis of the ERGIC 53 gene in 35 families with combined factor V-factor VIII deficiency
Blood 93: 2253-2260, 1999

Introduction

As outlined before information on the molecular lesions underlying recessive coagulation disorders is relatively scarce due to the rarity of these disorders. We thought that the study of Iranian patients had two main advantages: they are more frequent than in other countries and, being usually the result of consanguineous marriages, the gene defects in the two alleles are likely to be the same, facilitating screening and characterization. In the frame of the Iranian project, two defects were chosen to start the genotype analysis. The first is factor VII deficiency, because factor VII is a key factor placed at the intersection between the so called extrinsic and intrinsic coagulation systems. Therefore, it was thought that the natural mutations of the factor VII gene underlying factor VII deficiency might help to clarify the structure function relationship of the factor in blood coagulation. Moreover, factor VII deficiency it is the most frequent of the rare coagulation disorders, so that identification of the actual mutations is likely to be clinically useful in the Iranian population, should prenatal diagnoses become necessary in some of the women. We include in Section 3 of the thesis four papers, one that describes as a whole the factor VII gene mutations found in the Iranian patients (3.1) and three additional ones (3.2-4) that characterize in more details mutations of specific significance and novelty. The second defect chosen for this first part of the study was the combined deficiency of factors V and VIII, because at the time of starting this project no mutation underlying this disorder had yet been identified. In paper 3.5 we report in a collaborative study several new mutations in the ERGIC gene that associated with the combined factor V and VIII deficiency.

Molecular characterisation and three-dimensional structural analysis of mutations in 21 unrelated families with inherited factor VII deficiency

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Summary

Factor VII (FVII) is a four-domain glycoprotein that plays a critical role in the initiation of blood coagulation. Hereditary deficiencies of this plasma protein results in a bleeding diathesis that varies in severity amongst affected patients. We have analysed the FVII gene in 27 patients with FVII deficiency from 21 unrelated families predominantly of Middle-Eastern extraction.

A total of 19 different mutations were identified, of which 12 were novel and 7 had been previously reported. Nine of the 12 novel mutations were missense mutations located in the Gla domain (Ser23Pro), the second epidermal growth factor domain (Cys135Arg) and the catalytic serine protease domain (Arg247Cys, Arg277Cys, Ser282Arg, Pro-303Thr, Ser363Ile, Trp364Cys, Trp364Phe), of which five are homozygous. Three novel splice mutations were identified in intron 1a (IVS1a+5), intron 2 (IVS2+1) and intron 6 (IVS6+1).

Of the seven previously reported mutations, five were missense mutations of which three are homozygous (Gln100Arg, Arg152Gln, Arg304Gln, Cys310Phe and Thr359Met), one was a 17bp deletion (10585del17bp) and one was a splice site mutation within intron 7 (IVS7+7).

This study has significantly extended the current database of FVII mutations, including the number of known homozygous mutations. Conformational analyses of crystal structures for FVIIa and the FVIIa-tissue factor complex provided likely explanations for the effect of the missense mutations on FVIIa secretion or function. In particular, since 23 missense mutations were located to the serine protease domain, mostly to the region between the catalytic triad and the contact surface with tissue factor, this showed that the orientation of the serine protease domain relative to bound tissue factor in the complex is crucial for functional activity.

Introduction

Factor VII (FVII) is a vitamin K-dependent glycoprotein that plays a vital role in the initiation of coagulation. FVII is synthesised in the liver and circulates in the plasma as a single-chain zymogen at a concentration of 0.5 μg/ml (1). Following damage to the vascular endothelium, the cell surface receptor for FVII, tissue factor (TF), is exposed and forms a complex with FVII (2,3). Subsequent activation of FVII occurs through a proteolytic cleavage at Arg152-Ile153 to give a two-chain active serine protease, factor VIIa (FVIIa) (4). FVIIa is composed of a light chain (152 residues) and a heavy chain (254 residues) linked by a disulphide bond, and this directly activates factors IX and X to lead to the generation of thrombin (4-6).

FVII contains four independently-folded protein domains (4). The gene for FVII is 12.8 kb in length and located 2.8 kb 5' of the factor X gene on the long arm of chromosome 13. It contains 9 exons (1a, 1b, 2-8) and 8 introns, and its gene structure is broadly similar to those of factors IX and X, protein C and protein S (6). The alternative splicing of FVII mRNA leads to transcripts that encode

pre-pro leader sequences of either 38 or 60 amino acids, as this is encoded by exons 1a and 1b, and about 90% of the FVII transcripts in the liver lack exon 1b (7). Exons 2 and 3 encode parts of the propeptide and the Gla domain, whilst exons 4 and 5 encode the EGF-1 and EGF-2 domains (EGF: epidermal growth factor). Exons 6, 7 and 8 encode the catalytic serine protease (SP) domain. In the complex with FVIIa, the N-terminal fibronectin type III domain of TF makes contact with the EGF-1, EGF-2 and SP domains, and the C-terminal one makes contacts with the EGF-1 and Gla domains.

FVII deficiency is a rare, autosomally-inherited, recessive disorder that affects approximately 1 in 500,000 of the population. In contrast to many of the inherited bleeding disorders, there is a relatively poor correlation between the levels of FVII and the bleeding manifestations (8,9). Type 1 mutations correspond to when both the activity and antigen levels are proportionally reduced (i.e. when low levels of FVII occur in plasma), while Type 2 mutations correspond to when the antigen level of FVII is normal but the activity is reduced (dysfunctional). The most recent review of human FVII mutations lists 35 of these (10). The majority of these are missense ones, but in addition include a small number of deletions, splice site abnormalities and nonsense mutations. An updated list of FVII mutations is available on the Internet at http://europium.csc.mrc.ac.uk. In this study, the genetic and structural characterisation of 19 known and novel mutations in a group of patients of predominantly Middle-Eastern extraction with FVII deficiency is reported. Consanguineous marriage in such countries is common, and therefore the frequency of homozygous mutations is increased. The correlation of mutation sites with their location in a three-dimensional structure has potential to provide valuable molecular insights into function (11), especially if the mutations are homozygous and therefore are well defined in their clinical effect. Here, we show how analyses of the large number of mutations in terms of structures for FVIIa and its complex with TF (12-15) offer explanations for the decreased secretion or function of FVII. In particular, we show that the high concentration of mutation sites within the SP domain corresponds to a protein interface between the SP domain and TF which is likely to be crucial for the initiation of the thrombin formation pathway and is unable to tolerate evolutionary modification.

Patients and methods

Patients: 27 patients from 21 unrelated families and comprising 13 males and 14 females (age 5-58 yrs) with FVII deficiency were studied. Families were predominantly Iranian in origin (n=13), but included a smaller number from other countries, namely Italy (n=4), Malta (n=1), Pakistan (n=1) and the UK (n=1). Eleven out of 14 patients from Iran were the offspring of consanguineous marriages. Informed consent for investigation was obtained from all individuals.

To establish the type and severity of bleeding symptoms, we used a questionnaire which

was specially tailored to collect focused and objective information from patients (16). Severe bleeders were classified as those who had spontaneous and/or life-threatening bleeding episodes, such as hemarthroses, muscle hematomas, umbilical cord, gastrointestinal and central nervous system hemorrhages. Mild bleeders were patients who bled only after trauma or surgery or who had minor bleeding problems such as epistaxes or menorrhagia. Non-bleeders were those without any history of bleeding.

Factor VII assays: FVII coagulant activity (FVII:C) was measured in plasma using a one-stage prothrombin time-based assay and FVII deficient plasma (Dade International, Miami, Florida). Three types of thromboplastins were used to obtain FVII:C values, namely rabbit thromboplastin (OBT, Ortho Diagnostic Systems, Raritan, NJ), human recombinant thromboplastin (Recombiplastin, Ortho Diagnostic Systems) and bovine thromboplastin (Instrumentation Laboratory, Milan, Italy). FVII:C values were calculated using as standard pooled plasma from 40 normal individuals (20 men and 20 women not pregnant and not taking oral contraceptives). Standard plasma was assigned an arbitrary FVII:C value of 100%. Levels of FVII antigen (FVII:Ag) were measured using an enzyme immunoassay based on two murine monoclonal antibodies against FVII with epitopes on its light chain (17). The enzyme immunoassay based on these antibodies have been compared with a commercial enzyme immunoassay based on polyclonal antibodies and found to give comparable FVII:Ag values in parallel with known FVII expression levels (17). FVII:Ag levels were calculated using the same normal pooled plasma as the FVII:C standard.

DNA analysis: Genomic DNA was purified from peripheral blood leukocytes according to standard protocols. Following DNA extraction, the coding region, intron/exon boundaries and the untranslated regions of the FVII gene were amplified by polymerase chain reaction (PCR) and screened for mutations by single strand conformation polymorphism analysis (SSCP) (18) and conformation sensitive gel electrophoresis (CSGE) (19). Direct sequencing of amplified DNA was used to confirm and identify the presence of any mutations.

PCR: Amplification reactions were carried out in 100 µl volumes and comprised: 100-500 ng of DNA, 100 pmoles of each oligonucleotide primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 2 units of *Taq* polymerase. Reactions were denatured at 94°C for 5 min, then 40 cycles of amplification were performed. On the last cycle, the extension time was increased to 10 min. Oligonucleotide sequence data and amplification parameters are available on request.

Single stranded conformation polymorphism (SSCP) analysis: 1 µl of the final PCR product was mixed with 5 µl of denaturation buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue), denatured for 10 min at 94°C, then placed on ice for 2 min. 3-4 µl of each denatured sample was loaded onto a 10% polyacrylamide

gel (99:1 acrylamide: bisacrylamide) in Tris-Borate-EDTA buffer (0.09 M Tris-Borate, 2 mM EDTA) supplemented with 7.5% urea. Gels were pre-run at 700V for 60 min in Tris-Borate-EDTA buffer prior to loading, then electrophoresis was carried out at 450V for 12-15 h at room temperature (fragment size ~400 bp). DNA fragments were visualised by silver staining (Promega Corporation, Southampton, UK).

Conformation sensitive gel electrophoresis (CSGE): 5 µl of a wild-type control PCR product was mixed with 5 µl of patient PCR product and incubated at 98°C for 5 min and then at 65°C for 30 min to allow the formation of heteroduplexes. 5-8 µl of each sample was then mixed with 2 µl of loading buffer (40% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol) and 3-5 µl loaded onto a 10% polyacrylamide gel (99:1 acrylamide: bisacrylamide) containing 15% formamide and 10% ethylene glycol in Tris-Taurine-EDTA buffer (0.89 M Tris, 0.285 M Taurine, 2 mM EDTA). Gels were pre-run at 750V in Tris-Taurine-EDTA buffer for 60 min, the samples were loaded and electrophoresis carried out at 450V for 12-16 h. Following electrophoresis, gels were silver stained and photographed.

Sequence analysis: PCR products were purified (Bioline PCRapid Purification Kit, Bioline, London, UK) and directly sequenced using an ABI 377 automated sequencer (PE Applied Biosystems, Warrington, UK) with the same primers used for the amplification reactions. Any mutation identified by direct sequencing was confirmed by repeat sequencing and/or restriction enzyme digestion if associated with the creation or loss of a specific restriction enzyme site.

Structural analysis of FVII mutations: The crystal coordinates of FVIIa and the TF-FVIIa complex (12-14) were obtained from the Protein Data Bank (codes 1qfx, 1cvw and 1dan). Protein structures were visualised using the INSIGHT II 97.0 program package (Biosym/MSI, San Diego, USA) on Silicon Graphics INDY Workstations, using Crystal Eyes stereo glasses for detailed inspections. The quantitative assignment of the main-chain secondary structure of each structure was made using the DSSP program (20), where Figure 1 shows the α-helix and β-strand contents (denoted as H and E respectively) and other types including loops and turns (T, S, B, G). The quantitative assignment of solvent-exposed residue side-chains and the side-chains of residues that make contact between FVIIa and TF in the complex was made using the COMPARER program by the Lee and Richards method (21, 22) using a probe of 1.4 Å. Side-chains were defined as exposed if their accessibilities ranged between 20-100% (values of 2 to 9 in Figure 1), and buried if these ranged between 0-19% (values of 0 and 1 in Figure 1).

Results

Phenotypic analysis: Table 1 shows the FVII:C and FVII:Ag levels for each of 27 patients from 21 unrelated families and the severity of their bleeding symptoms. Only FVII:C data obtained with recombinant

human thromboplastin are shown. 11 out of 27 patients showed reduced levels of both FVII:C and FVII:Ag, and these are assigned as type 1 defects. In the remaining 16 patients, there was a low level of FVII:C and a normal or borderline level of FVII:Ag and these were assigned to the presence of a dysfunctional FVII molecule (type 2 defects). In 22 out of 27 patients, similar FVII:C values were obtained using three different thromboplastins (human, bovine and rabbit). However, five Iranian patients from two unrelated families (Pedigrees J and K) had low FVII:C levels with rabbit thromboplastin (<1%), mildly reduced levels with human thromboplastin (17-37%) and normal levels with bovine thromboplastin (70-130%). These five patients had normal or elevated levels of FVII:Ag. A total of 19 mutations were identified, of which 12 were novel and 7 had been previously reported (Table 2).

Novel Mutations: Nine of the 12 novel mutations were missense mutations, and three were splice-site mutations. Of the 12 families in which novel mutations were identified, seven were homozygous (Pedigrees F, H, Q-U) and the remaining five were heterozygous (Pedigrees A, C, E, G and V). All but two of the missense mutations occurred in the catalytic SP domain of FVII. One non-SP domain mutation was Ser23Pro in the Gla domain that was found in a single patient (Pedigree A) who was heterozygous for the mutation. Although FVII:Ag is close to half-normal in this individual, as expected for a heterozygote in which no other FVII mutation or gene could be identified, FVII:C was less than 1% to indicate a dysfunctional type 2 defect. The other non-SP domain mutation was Cys135Arg, which occurs in the EGF-2 domain of FVII in patient C-1. This patient was heterozygous for this mutation, which can be a type 1 defect as both FVII:C and FVII:Ag were reduced (but see below). A second previously-reported 17 bp deletion mutation within exon 8 (10585del17) (23) was also identified in patient C-1. Of the seven novel SP domain mutations, four were homozygous and associated with a normal FVII:Ag level but reduced functional activity, indicating dysfunctional type 2 defects (Pedigrees H, Q-S). In the remaining three homozygous or heterozygous cases, there was a reduction in FVII function and a partial or full reduction in the FVII:Ag level, indicating type 1 or 2 defects (Pedigrees E-G).

The remaining 3 novel mutations were splice sites. In Pedigree T, a homozygous G->A transition within intron 1a (IVS-1a G5A) was associated with a parallel reduction in both FVII:Ag and FVII:C. In Pedigree U, a homozygous G->A substitution within intron 2 (IVS-2 G1A) was associated with a severe bleeding diathesis and undetectable levels of FVII:Ag and FVII:C. In Pedigree V, a heterozygous G->T substitution was identified within intron 6 (IVS-6 G1T). There is a disparity between FVII:Ag (30%) and FVII:C (<1%) in this case, which suggested the presence of an additional mutation that has not been identified.

Previously reported mutations: Of the 7 previously identified mutations, Arg304Gln

(23,24), Cys310Phe (24,25) and Thr359Met (26) correspond to the SP domain, however the Thr359Met mutation is now homozygous (Pedigree P; type 1 defect), and not heterozygous as previous. The Gln100Arg and Arg152Gln mutations (27,28) in the EGF-2 domain are homozygous in Pedigree B (type 1 defect) and Pedigree D (type 2 defect), and not heterozygous as previous. The IVS-7 splice site mutation (29) was found in a single individual (Pedigree W), who was heterozygous for the substitution, in which the FVII:C level were consistent with a single causative mutation.

Tab. 1 - **Phenotypic and clinical data on 27 patients with FVII deficiency**

Kindred/ No.	FVII:C* (50-150%)	FVII:Ag (70-120%)	Consanguinity	Bleeding Symptoms
A-1	<1%	77%	No	Severe
B-1	<1%	10%	No	Severe
C-1	<1%	<1%	No	Severe
D-1	<1%	78%	Yes	Mild
E-1	<1%	54%	No	Severe
E-2	<1%	43%	No	Mild
E-3	<1%	43%	No	Mild
F-1	<1%	<1%	Yes	Severe
G-1	<1%	6%	No	Severe
H-1	<1%	135%	Yes	Severe
J-1	37%	240%	Yes	None
J-2	27%	224%	Yes	Mild
J-3	24%	156%	Yes	None
J-4	17%	106%	Yes	None
K-1	22%	130%	Yes	None
L-1	<1%	129%	Yes	Mild
M-1	<1%	80%	Yes	Mild
N-1	<1%	104%	Yes	Mild
P-1	<1%	<1%	Yes	Mild
Q-1	<1%	80%	Yes	Mild
Q-2	<1%	130%	Yes	Mild
R-1	<1%	132%	Yes	Severe
S-1	6%	77%	Yes	Severe
T-1	6%	3%	Yes	Mild
U-1	<1%	<1%	Yes	Severe
V-1	<1%	30%	No	Mild
W1	31%	100%	No	None

*VII:C obtained using recombinant human thromboplastin (tissue factor).

Discussion

We have used two screening methods combined with direct sequencing of PCR products to identify 19 candidate mutations in the human FVII gene in 27 kindreds, of which 12 were novel ones (Table 2). When combined with the most recent review of 35 FVII mutations (10), this makes a total of 47. The FVII mutation website currently lists 70 mutations (February 2000), including subsequent examples of 3 of our 12 novel mutations (30,31). The number of novel mutations presented here is the largest of any single study to date, and increases the known total to 79. Since 5 novel mutations and 3 previously known mutations are homozygous, Table 1 clearly identifies the clinical consequence of these mutations (Pedigrees B, D, F, H and P-S). In order to explain the likely effect of the missense mutations on FVII, we quantitatively identified the secondary structures and solvent

Tab. 2 - **Mutational data on 27 patients with FVII deficiency**

Kindred/No	Mutation	Codon	Exon	Domain	Comments
A-1	TCC-CCC	Ser23Pro	2	Gla	Heterozygous
B-1	*CAG-CGG*	*Gln100Arg**	5	EGF-2	Homozygous
C-1	10585del17	223-229*	8	SP	Compound
	TGT-CGT	Cys135Arg	5	EGF-2	heterozygote
D-1	*CGA-CAA*	*Arg152Gln**	6	SP	Homozygous
E-1	CGC-TGC	Arg247Cys	8	SP	Compound
	TGC-TCC	*Cys310Phe**		SP	heterozygote
E-2	CGC-TGC	Arg247Cys	8	SP	Compound
	TGC-TCC	*Cys310Phe**		SP	heterozygote
E-3	CGC-TGC	Arg247Cys	8	SP	Compound
	TGC-TCC	*Cys310Phe**		SP	heterozygote
F-1	CGC-TGC	Arg277Cys	8	SP	Homozygous
G-1	AGC-AGG	Ser282Arg	8	SP	Heterozygous
H-1	CCC-ACC	Pro303Thr	8	SP	Homozygous
J-1	*CGG-CAG*	*Arg304Gln**	8	SP	Homozygous
J-2	*CGG-CAG*	*Arg304Gln**	8	SP	Homozygous
J-3	*CGG-CAG*	*Arg304Gln**	8	SP	Homozygous
J-4	*CGG-CAG*	*Arg304Gln**	8	SP	Homozygous
K-1	*CGG-CAG*	*Arg304Gln**	8	SP	Homozygous
L-1	*TGC-TCC*	*Cys310Phe**	8	SP	Homozygous
M-1	*TGC-TTC*	*Cys310Phe**	8	SP	Homozygous
N-1	*TGC-TTC*	*Cys310Phe**	8	SP	Homozygous
P-1	*ACG-ATG*	*Thr359Met**	8	SP	Homozygous
Q-1	AGC-ATC	Ser363Ile	8	SP	Homozygous
Q-2	AGC-ATC	Ser363Ile	8	SP	Homozygous
R-1	TGG-TGC	Trp364Cys	8	SP	Homozygous
S-1	TGG-TTT	Trp364Phe	8	SP	Homozygous
T-1	Cgt-cat	IVS1a+5	IVS-1a	-	Homozygous
U-1	Gtga-atga	IVS2+1	IVS-2	-	Homozygous
V-1	Ggta-gtta	IVS6+1	IVS-6	-	Heterozygous
W-1	*Gtac-gtag*	*Splice site**	IVS-7	-	Heterozygous

* *Previously reported mutations.*

accessibilities of residues in experimentally-determined crystal structures for free FVIIa and the TF: FVIIa complex (12-14) (Figure 1). That these crystal structures are good representations of the multidomain structures in solution was shown by scattering modelling calculations (15).

Structural interpretation of the novel mutations: The catalytic SP domain consists of two subdomains, SP1 and SP2, each of which contains a four-stranded Greek key-sheet motif and a two-stranded-hairpin motif. The catalytic triad H193-D242-S344 is positioned between SP1 and SP2 (Figure 3). Six of the 12 novel mutations occur in the SP2 subdomain that interacts directly with TF, two occur in the Gla and EGF-2 domains, one occurs in the SP1 subdomain, and three are splice site mutations (Table 2).

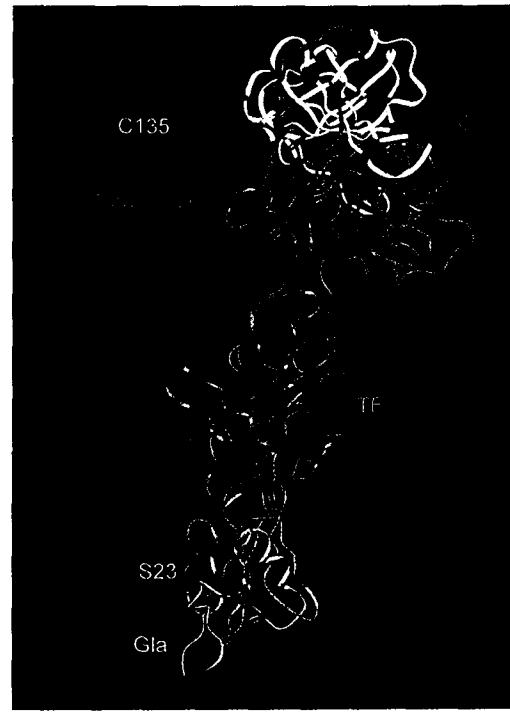
The Ser23Pro substitution in the Gla domain results in a dysfunctional defect (Figure 2). The second mutant allele in this heterozygous patient has not been found by PCR-based mutation screening. Ser23 is conserved in the Gla domains of factors IX and X, and this mutation replaces a small exposed hydrophilic residue (Figure 1) by a bulky hydrophobic one with a restricted main-chain conformation. As the FVII:Ag

sent in heterozygous association with a 17 bp deletion mutation in exon 8 which prevents synthesis of FVII. Cys135 is disulphide-bridged with Cys262 in the FVII structure, and connects the light and heavy chains of FVIIa after the Arg152-Ile153 peptide bond is cleaved during activation. The physical separation of the SP domain and light chain would lead to the complete lack of functional activity of FVII. Even though it is not clear whether the low FVII:Ag assay corresponds to the rapid clearance of FVII from plasma, a low level of secretion in a misfolded FVII molecule, or a lack of epitope recognition in assays, it is not surprising that the two mutations in this patient results in a severe FVII deficiency and bleeding tendency (Table 1).

The Arg247Cys mutation is the only novel mutation that is located in the N-terminal subdomain SP1 which makes no contacts with TF in the complex (Figures 2 and 3), so is unlikely to correspond to a dysfunctional type 2 defect. In Pedigree E, this mutation occurs in association with a previously-described homozygous mutation (Cys310Phe) (10,24,25) in the SP2 subdomain (Figure 3: see below). Since the Cys310Phe mutation results in a type 2 loss of function in the homozygous Pedigrees L, M and N, the reduction to half-normal FVII levels in Pedigree E shows that the Arg247Cys mutation is likely to be a type 1 defect. The side-chain of Arg247 is in the protein core, being only 10% accessible and within β-strand H (Figure 1). The introduction of a Cys residue in this mutation may perturb any of the 12 Cys-Cys bridges within FVII to affect the correct folding of FVII. This would cause either a lack of secretion of FVII or a short half-life in plasma, and is consistent with the assignment of a type 1 defect to the Arg247Cys mutation.

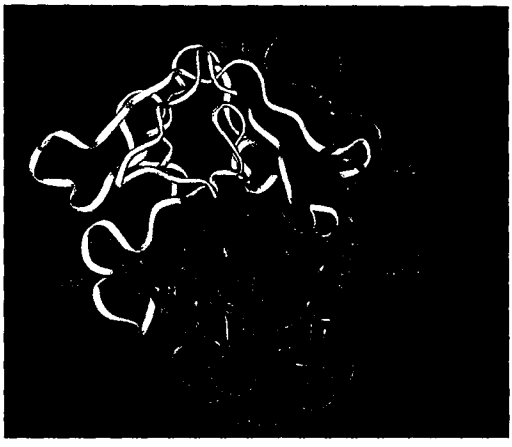
A homozygous substitution Arg277Cys was

Fig. 2 - **Ribbon view of the crystal structure of the complex between FVIIa and TF.**



The α-carbon atoms of the novel mutation sites (yellow; Table 2) and previously-identified ones (blue: Table 2) and the catalytic triad (red: Figure 1) are represented by spheres. The domains in FVIIa are identified as follows: Gla, dark grey; EGF-1 and EGF-2, green; the N-terminal and C-terminal SP subdomains, light grey and magenta respectively. The two fibronectin type III domains in tissue factor are identified in brown.

Fig. 3 - Ribbon view of the main-chain of the catalytic SP domain of FVIIa.



The α-carbon atoms of the catalytic triad H193-D242-S344 are shown as red spheres at the interface between the two subdomains of the SP domain (light grey: upper N-terminal subdomain; magenta: lower C-terminal subdomain). The novel mutation sites of this study are shown in yellow, while previously-identified ones also found in this study are shown in blue (Table 2).

identified as a type 1 defect in an Iranian patient with a severe bleeding diathesis (Table 1). The introduction of a Cys residue may cause protein misfolding and affect FVII secretion or reduce its half-life in plasma. The substitution of this residue during alanine scanning mutagenesis studies showed a combined impairment of both TF binding and proteolytic activity (33), which identifies this to be a type 1 defect. In the crystal structure of the complex, Arg277 is packed against Trp45 of TF and forms hydrogen bonds with the Ser39 sidechain and the Gly43 mainchain in TF (14). Unsurpri-singly, the solvent accessibility of Arg277 is much reduced from 70% in free FVIIa to 10% in the complex (Figure 1).

The Ser282Arg mutation involves a residue conserved in both factors IX and X, and corresponds to a heterozygous type 1 defect. The mutation occurs in an Iranian boy with a severe bleeding disorder, in whom the mutation on the other allele has not been identified by PCR-based mutation screening. Ser282 lies in the protein core of the SP2 subdomain of FVII within β-strand J with 0% solvent accessibility (Figure 1). It is close to the N-terminal residue Ile153, whose charged amino group forms an internal salt bridge with Asp343 in the oxyanion pocket next to Ser344 of the catalytic triad (Figure 3). The mutation of a small buried residue by a large basic one is likely to have significant steric or electrostatic effects on the oxyanion pocket. This will affect the folding of the SP domain, resulting in a low secretion or short plasma half-life for FVII.

The Pro303Thr mutation is a type 2 dysfunctional homozygous substitution in an Iranian patient with a history of recurrent and severe bleeding. Pro303 is conserved in factors IX and X, the complement serine proteases (factor D, C1r and C1s), and elastase and trypsin from several species (15,34). Pro303 is buried with only 10% solvent accessible in the SP domain in β-strand K, and may stabilise an adjacent turn in the protein backbone (Figure 1). In the FVIIa-TF complex, Pro303 is close to TF in the region where the TF residues Gly, Ser, Ala and Thr at positions 1-4 and 85-90 are absent from the crystal structure. It is likely that Pro303 is important for the stabilisation of the surface of the

SP domain in the complex, and this would explain the severity of the Pro303 substitution by Thr (Table 1). The adjacent residue Arg304 directly interacts with TF, however its mutation has only a mild effect on FVIIa function (see below). In confirmation of this explanation, site-directed mutagenesis of Pro303 and Arg304 showed that they are important to stabilise the complex and the proteolytic function of FVIIa (33).

Three dysfunctional mutations occur at residues 363 and 364. The Ser363Ile mutation is homozygous in 2 members of an Iranian family with definite bleeding histories. In each case, the FVII:Ag levels were preserved but there was a reduction in FVII:C to less than 1%. Two homozygous mutations were identified at residue 364, one being Trp364Cys, and the other is a two-nucleotide substitution that gives Trp364Phe. Both mutations were associated with preservation of FVII:Ag but a reduction in FVII:C and a severe effect on bleeding. Ser363 and Trp364 are conserved residues in SP domains (15,34) and occur at the C-terminus of the β-strand N at the active site cleft. As both residues form four mainchain hydrogen bonds to the active site substrate analogues D-Phe-L-Phe-Arg chloromethyl ketone or 1,4-dansyl Glu-Gly-Arg chloromethyl ketone (13,14), the effect of these mutations is most likely due to perturbations in the substrate-binding site of FVIIa, in accordance with its type 2 classification.

In relation to the three novel splice site mutations, the homozygous IVS1a+5 mutation results in the preservation of some FVII coagulant activity and is associated with a mild bleeding history. In contrast, the homozygous IVS2+1 mutation is associated with major bleeding problems (intracranial haemorrhage, muscle haematomas) and a FVII:Ag and FVII:C of less than 1%. A similar mutation at the 5' splice site of intron 4 (IVS4+1) results in the excision of the EGF-1 domain of FVII, and is associated with a complete absence of circulating FVII (35). It is probable that a similar mechanism operates in the case of the IVS2+1 mutation. The third mutation, IVS6+1, is heterozygous, in which the FVII:C of less than 1% suggests the presence of an additional unidentified mutation. Despite a low FVII activity, the clinical phenotype was mild.

Structural interpretation of previously identified mutations: A review of 35 previously-described mutations (10) showed that 26 corresponded to amino acid substitutions found in the Gla (1 mutation), EGF-1 (3 mutations) and EGF-2 (5 mutations) domains, and the SP1 and SP2 subdomains (5 and 12 mutations respectively). Seven of these missense mutations were also identified in the 27 patients of the present study (Gla, 1 mutation; EGF-2, 2 mutations; SP2, 4 mutations), with an important difference in that three have now been identified in homozygous forms. The earlier discussion of structure-function aspects (10,36) can now be extended in the light of recent crystal structures (12-14) and their known homozygous or heterozygous forms.

The homozygous Gln100Arg mutation in the EGF-2 domain, previously known only in

heterozygous form (10), involves the substitution of a 0% accessible residue (Figure 1) conserved in factors IX and X by a large positively charged residue. Gln100 is located close to the junction of the EGF-2 domain with the SP domain (Figure 2), so the substitution is likely to perturb the protein folding of FVIIa in this critical region. This interpretation would be consistent with the assignment of this mutation as a type 1 defect (Table 1).

The homozygous Arg152Gln substitution adjacent to the SP domain coincides with the factor Xa cleavage site at Arg152-Ile153 in FVII. This would prevent the cleavage and activation of FVII, which is otherwise unaltered, so this is consistent with its type 2 assignment. Previously, this mutation had been observed in homozygous association with a second one at Arg79Gln in the EGF-1 domain, where Arg79 is conserved in factors VII, IX and X (10,28). As this second mutation was absent in Kindred D-1, the clinical effect of this mutation can now be defined.

In the SP2 subdomain, the dysfunctional type 2 mutation at Arg304Gln involves a CpG dinucleotide, a recognised hotspot for mutation in the human genome. This substitution is homozygous in 2 Iranian families (Pedigrees H and N) and has been previously reported in families from 7 additional countries. The different FVII coagulant activities measured with TF from different species is characteristic of this mutation (FVII Padua 1), and presumably arises from variations in the TF sequences that correspond to the contact area between FVIIa and TF (37,38). The crystal structure of the complex shows that Arg304 binds to three H_2O molecules within a hydrophilic pocket between FVIIa and TF. The likely effect of this mutation is to perturb this water structure between the two protein surfaces, thus explaining its type 2 effect.

In the SP2 subdomain, the Cys310Phe substitution was found in 3 unrelated families (Pedigrees L-N) as a homozygous mutation, and in another one (Pedigree E) that was compound heterozygous for this and a second mutation Arg247Cys (see above). In the homozygous families, laboratory measurements showed normal FVII:Ag but a reduction in FVII:C to less than 1%, indicating a dysfunctional defect. Cys310 is highly conserved in all serine proteases, including factors IXa and Xa, and is 0% solvent accessible in α-helix A2 (Figure 1). It forms a disulphide bond with Cys329 near the surface of the SP domain in the only potentially mobile segment between the contact surfaces of FVIIa and TF (Figure 3) (14). The reduction of this disulphide bond severely impairs the interaction with TF (39). The type 2 assignment is best explained in terms of the inclusion of a bulky hydrophobic residue inside the SP domain that has altered the FVII structure in the region where it forms contacts with TF.

The Thr359Met mutation is a homozygous type 1 defect that previously had only been seen as a heterozygote (10), so its clinical phenotype has now been defined here. In the SP2 subdomain, the incorporation of a bulky hydrophobic residue at a buried location in a

central β-strand N is expected to affect the packing of residues within the SP2 subdomain. Since Thr359 is packed against the surface α-helix A1, its substitution by Met is expected to alter the position of this α-helix, and the resulting misfolding may lead to low secretion or a short plasma half-life.

Conclusion

The large number of FVII mutations presented here, including many homozygous ones, has significantly enlarged our knowledge on gene mutations associated with FVII deficiency, the most frequent of the rare recessively-transmitted coagulation disorders. In the absence of the verification of each FVII dysfunction using expression work based on each missense mutation for functional assays and crystallisation studies, views of the experimentally-determined crystal structures of free and complexed FVIIa were used to explain the effect of each mutation. *In vitro* expression analyses are under way to further characterise the effects of the novel mutations. The identification of 12 novel and 9 known mutations in this study leads to a total of 79 known mutations in FVII (Figure 1; Tables 1 and 2), from which three significant conclusions can be made:

(•) The novel Arg277Cys, Pro303Thr, Ser363Ile, Trp364Cys and Trp364Phe mutations are homozygous, and their laboratory phenotypes permitted the identification of Arg277Cys as a type 1 quantitative defect and the other four as type 2 dysfunctional defects. The Gln100Arg, Arg152Gln and Thr359Met mutations were found to be homozygous ones instead of heterozygous previously, and can be identified as type 1, type 2 and type 1 defects respectively.

(•) The quantitative comparison of the single-residue mutations with crystal structures for FVIIa and the FVIIa-TF complex provided good explanations for the observed type 1 and 2 classifications, with only one ambiguity in the case of Arg277Cys. These explanations clarify the assignments of the laboratory phenotypes. The type 1 defects were attributable to misfolded proteins that fail to be secreted or have short half-lives in plasma. The type 2 defects correlate with normal levels of circulating FVIIa but involve residues close to functionally important regions of FVIIa, such as at the protein surface in contact with TF in the FVIIa-TF complex.

(•) The location of the mutations in the FVII structure provides information on its molecular function. All four domains in FVII contribute to TF binding, where each domain contributes approximately equally to about 21% to 31% of the area in contact with TF. Functionally-defective mutations are therefore expected to occur at evolutionarily-similar rates in the four domains. This is however not observed, where the addition of 9 novel missense mutations in this study to those reviewed in (10) gives a total of 35, of which half originates from the SP2 subdomain, and a quarter from the EGF-2 domain. It is generally thought that the association of FVIIa with TF locks the SP domain into a proteolytically-active conformation. Our localisation of defective mutations from this study provi-

des evidence that the most important conformational changes during FVII activation are those within the C-terminal SP2 subdomain of the SP domain that makes contact with TF and leads to FVII activation, and that residue conservation is critical for this. The summary provided in Figure 1 will be useful for the classification of further FVII mutations to be identified.

Acknowledgements

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Homozygous 2bp deletion in the human factor VII gene: a non-lethal mutation that is associated with a complete absence of circulating factor VII

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Abstract

We report the case of a 5-year-old boy with severe factor VII deficiency. The affected child presented at the age of 8 months and again at 18 months with bleeding from the gastrointestinal tract but the diagnosis of factor VII deficiency was not made until the age of 3 years. He was treated with fresh frozen plasma and subsequently factor VII concentrates and to date remains well. To identify the causative mutation, the factor VII gene was screened by SSCP and direct sequence analysis. A single homozygous 2bp deletion (-CT) mutation was identified in exon 1a removing nucleotides 27/28 (codons 52/53). Both parents, who were first cousins, were heterozygous for the mutation. The mutation located in the prepropeptide of factor VII, results in a complete absence of factor VII in plasma. This case indicates that a complete absence of plasma factor VII is not necessarily a lethal condition.

Introduction

Human factor VII is a vitamin K-dependent glycoprotein of 406 amino acids (1). It is synthesised by the liver and circulates in plasma as a single-chain form with a concentration of ~0.5 μg/ml. The gene for factor VII maps to chromosome 13 (13q34) and consists of 9 exons (exons 1a, 1b, 2 —> 8) and 8 introns spanning 12.8 kb of sequence (2, 3). Factor VII consists of several discrete functional domains that include the Gla domain, two epidermal-like growth factor domains and a catalytic domain (2).

Factor VII is crucial to the initiation of blood coagulation (4,5). Damage to the vascular endothelium exposes tissue factor (TF)-the cellular receptor for factor VII. Exposed TF rapidly binds factor VII resulting in the formation of a TF-VII complex. Factor VII bound to TF undergoes a proteolytic cleavage (at Arg152-Ile153) resulting in the two-chain active serine protease -factor VIIa. Factor VIIa then activates factors IX and X leading to the generation of thrombin.

Congenital factor VII deficiency is a rare autosomally inherited, recessive bleeding diathesis that affects approximately 1:500,000 individuals (6). Factor VII deficiency, in common with many other inherited recessive disorders is more frequent in countries where consanguineous marriage occurs (7). In contrast to haemophilia A or B in which the bleeding tendency correlates very well with the factor VIII or IX level, there is a relatively poor correlation in factor VII deficiency between factor VII levels and the bleeding tendency, for reasons which are unclear.

The possibility that a complete absence of factor VII in plasma is incompatible with life is suggested by the paucity of mutations that can be confidently predicted to result in a total lack of circulating factor VII. McVey and colleagues recently reported a 5' splice site mutation within intron 4 of the factor VII gene that leads to exon skipping and the deletion of exon 4 from factor VII mRNA (8). *In vitro* expression studies failed to demonstrate any produc-

tion of factor VII and the authors concluded, therefore, that the presence of this homozygous mutation was associated with a complete absence of factor VII.
The affected child died from massive intracranial haemorrhage at the age of 12 days.
In this study we report for the first time a novel non-sense homozygous mutation located in exon 1a of the factor VII gene, coding for the prepropeptide sequence. This mutation leads to a complete absence of plasma factor VII but the patient remains alive.

Methods

Coagulation assays: Blood was collected into 3.8% trisodium citrate in a ratio of 1 part anticoagulant to 9 parts blood. Prothrombin times were performed using recombinant human tissue factor (Recombiplastin, Ortho Diagnostic Systems, Milan). Factor VII coagulant activity was assayed in plasma using a one-stage clotting assay based upon recombinant human tissue factor and factor VII deficient plasma. This method measures factor VII activity levels as low as 0.01u/ml (9). Factor VII antigen was measured using an immunoradiometric assay based upon the use of two previously described anti-human factor VII mouse monoclonal antibodies (10). This method can measure factor VII antigen levels as low as 0.0001u/ml.
DNA amplification: DNA was isolated from peripheral blood leukocytes and the 9 exons of the factor VII gene together with the flanking intronic sequences, amplified by polymerase chain reaction.
Amplification reactions were carried out in 100 µl volumes and comprised: 100-500 ng of DNA, 100 pmoles of each oligonucleotide primer, 200µM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 2 units of *Taq* DNA polymerase. Reactions were denatured at 94°C for 5 minutes and then 40 cycles of amplification performed. On the last cycle the extension time was increased to 10 minutes. (Oligonucleotide sequence data and amplification parameters are available on request.)
Single strand conformation polymorphism analysis (SSCP) and sequence analysis: PCR samples were denatured by mixing with 2 volumes of denaturation buffer (95% formamide, 0.05% xylene cyanole and 0.05% bromophenol blue) and incubating at 94°C for 10 minutes and then rapidly chilling on ice for 2 minutes. 4µl of each sample was then loaded onto a 10% polyacrylamide gel (99:1 acrylamide: bisacrylamide) containing 7.5% urea and 1X Tris-Borate-EDTA (TBE). Gels were pre-run at 700V for 60 minutes at room temperature in 1X TBE prior to loading the samples.
Electrophoresis was carried out at 450V for 12-15 hours - the precise times were dependent upon the size of the PCR fragments being analysed. Following electrophoresis, DNA fragments were detected by silver staining (Promega Biotechnology, UK) and any variant bands identified.

Tab. 1 - **Results of Factor VII assays**

	Factor VII Antigen (Normal range: 0.8-1.2u/ml)	Factor VII Activity (Normal range: 0.8-1.2u/ml)
Index	<0.0001	<0.01
Mother	0.56	0.53
Father	0.52	0.43

Direct sequencing of PCR products was performed using an Applied Biosystems 377 DNA sequencer with the amplification primers employed as sequencing primers.

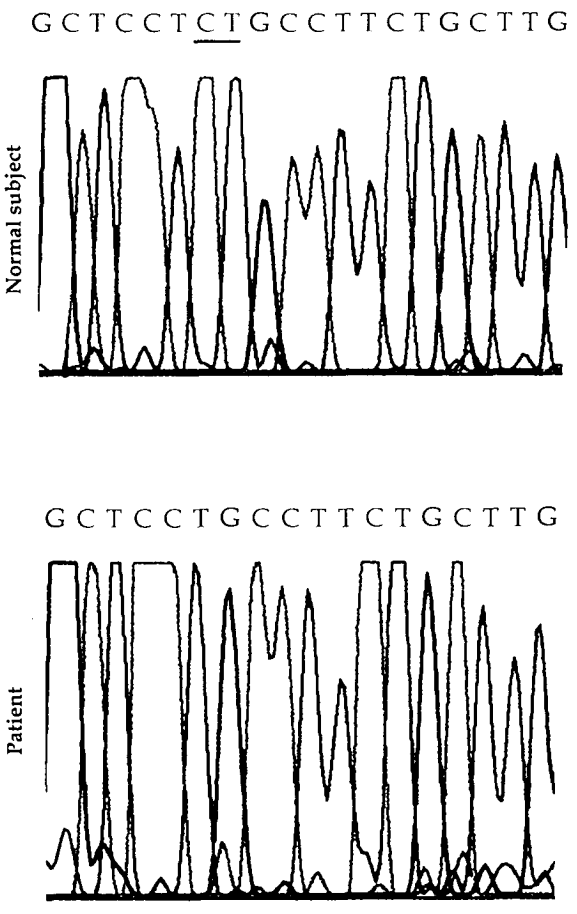
Results

Case history and coagulation studies: The index case, the offspring of a consanguineous marriage between first cousins, is a 5-year-old-male of Chinese origin. The child was born by normal vaginal delivery and there were no developmental problems associated with the neonatal or perinatal periods. At the age of 8 months and again at 18 months he presented with severe bleeding from the gastrointestinal tract. However, the diagnosis of factor VII deficiency was not made until the age of 3 years when he presented with recurrent bleeding problems-haematuria, haemarthroses, muscle and soft tissue haematomas and bleeding from the gastrointestinal tract. Many of these bleeding problems were spontaneous or occurred in relation to minor trauma. Investigations showed a prolonged prothrombin time, a normal activated partial thromboplastin time and markedly reduced factor VII activity and antigen levels at less than 0.01u/ml and 0.0001u/ml respectively, consistent with severe factor VII deficiency (Table 1). Screening of the father and mother showed factor VII activity levels of 0.43u/ml and 0.53u/ml and factor VII antigen levels of 0.52u/ml and 0.56u/ml respectively. The proband has four adolescent sisters but all have declined to have their factor VII levels measured. There was no family history of a bleeding diathesis.

Following diagnosis, treatment was instituted with fresh frozen plasma (FFP) with a maximum daily dose of 2 units every 12 hours. The patient received a total of 301 units of FFP and 6 units of packed red cells over the subsequent 12 months reflecting the severity of his bleeding diathesis. As a result of the problems of circulatory overload associated with the use of large volumes of FFP, replacement therapy with a plasma-derived factor VII concentrate was commenced at a dose of 500u (32u/kg) every 12 hours. Following the introduction of factor VII concentrates, his bleeding problems rapidly resolved. After 5 months of prophylactic treatment, factor VII concentrates were stopped and further treatment was given whenever the child was symptomatic. To date he remains well.

Mutational analysis: To identify the mutation in this family, the entire coding region of the factor VII gene was amplified and screened for mutations by SSCP. SSCP analysis revealed a mobility difference between control and patient in PCR fragments that encompassed exon 1a. Furthermore, the absence of a normal pattern of bands suggested that this was a

Fig. 1 - **Nucleotide sequence of exon 1a.**



(A) Normal sequence - the 'CT' dinucleotide that is deleted is underlined. (B) Sequence from the index case showing a homozygous deleted 'CT' dinucleotide.

homozygous mutation. Direct sequence analysis of this region revealed the index case to be homozygous for a 2bp deletion (nucleotides 27/28:-CT) in exon 1a at codon -52/51 (Figure 1), a region that encodes part of the prepropeptide of factor VII. Sequence analysis of both parents confirmed that they were both heterozygous for the 2bp deletion mutation.

Discussion

This paper de-scribes a novel mutation in the human factor VII gene that is associated with undetectable circulating plasma factor VII. The mutation is a 2bp deletion (CT) in exon 1a (codon-52/-51) and is located in the prepro-leader sequence of factor VII. Factor VII is synthesised with a prepro-leader sequence of either 60 or 38 amino acids, a difference which arises from alternative splicing of exon 1a/1b (11). Ap-proxima-tely 90% of factor VII mRNA transcripts lack exon 1b and contain only exon 1a. In transcripts which exclude exon 1b, the 2bp deletion results in a frameshift and the creation of a premature stop codon at residue 83 conserving only the N-terminal 9 amino acids of the normal prepropeptide se-quence. The remaining 74 amino acids encoded by this sequence share no homology with any of the mature factor VII protein or indeed of the other vitamin K dependent clotting factors. In contrast, in the small number of transcripts that include both

exon 1a and 1b, the mutation results in the creation of a stop codon at position 25 but again only the N-terminal 9 amino acids of the normal propeptide sequence are conserved.

Our current understanding of blood coagulation in man suggests that a failure to initiate coagulation through the binding of tissue factor to factor VII is likely to be incompatible with life. This theory is supported by the recently described factor VII knockout mouse which, although developing normally to term, dies shortly at/or after birth from major abdominal and intracranial haemorrhage (12). In addition, in the case reported by McVey and colleagues (8) a homozygous splice site mutation in the factor VII gene appeared to be associated with a complete absence of factor VII resulting in a fatal intracerebral haemorrhage at the age of 14 days. The authors, on the basis of this data, have concluded that the complete absence of factor VII is incompatible with life (8)

In the case we have described, bleeding symptoms did not develop until the age of 8 months and the diagnosis was not made until the age of 3 years, although the subsequent coagulation data were consistent with severe factor VII deficiency. It is unclear why there is such a marked difference in clinical manifestations between this case and that described by McVey and colleagues (8) or the factor VII knockout mouse model. The possibility that a mechanism might exist to partially correct the 2 bp deletion mutation was considered. Transcriptional errors restoring the reading frame and/or ribosomal slippage during translation, have been shown to partially correct the molecular defect in severe haemophilia A and other inherited disorders, resulting in a less severe clinical phenotype (13,14). Such mutations have, to date, involved the deletion of only a single nucleotide that occurs in a sequence of identical bases. In addition the correction results in the presence of functionally active protein. The 2 bp deletion within the factor VII gene reported in this paper involves non-identical nucleotides and does not occur in a run of similar bases. In addition, the absence of any detectable factor VII antigen in plasma of the index case using a sensitive IRMA assay (capable of detecting factor VII antigen as low as 0.0001u/ml), would argue against this hypothesis. A second mutation within the factor VII gene to correct the frame-shift was also considered. However, such a mutation would have to occur within the pre-pro-peptide to allow efficient post-translational modification and subsequent export of the protein.

It is possible that some alternative mechanism exists that in part corrects the clinical phenotype. This may involve the activation of coagulation through factor XII or through some novel serine protease but at present this is speculation.

In summary, we report the first case of a homozygous mutation in the prepropeptide coding region of the factor VII gene. This 2 bp deletion mutation leads to the creation

of a premature stop codon, and to the complete absence of factor VII activity and antigen. In contrast with a recent publication by McVey (8) our finding indicates that such a mutation resulting in an absence of factor VII in plasma, is not incompatible with life.

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Characterization of two naturally occurring mutations in the second epidermal growth factor-like domain of factor VII

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Abstract

We investigated the mechanisms responsible for severe factor VII (FVII) deficiency in homozygous Italian patients with either $Gly^{97}Cys$ or $Gln^{100}Arg$ mutations in the second epidermal growth factor domain of FVII. Transient expression of complementary DNA coding for the mutations in COS-1 cells showed impaired secretion of the mutant molecules. Using stably transfected Chinese hamster ovary (CHO) cells, we performed pulse-chase labeling studies, immunohistochemistry, and experiments with inhibitors of protein degradation, showing that $FVII\text{-}Cys^{97}$ did not accumulate intracellularly but was degraded in a pre-Golgi, nonlysosomal compartment by a cysteine protease. In stably transfected CHO cells expressing $FVII\text{-}Arg^{100}$, the level of intracellular FVII was not increased by several inhibitors of protein degradation, but $FVII\text{-}Arg^{100}$ was retained in the endoplasmic reticulum for a longer period of time than wild-type FVII. $FVII\text{-}Arg^{100}$ had a lower apparent molecular weight than did wild-type FVII under nondenaturing conditions, which is attributable to misfolding due to abnormal disulfide bond formation.

Introduction

Human factor VII (FVII) is a vitamin K-dependent glycoprotein that normally circulates in plasma at a concentration of 0.5 μg/mL.(1) FVII in association with tissue factor initiates blood coagulation by activating factor IX and factor X(2,3). The mature FVII molecule is a single chain of 406 amino acids and is comprised of several discrete domains including the Gla domain, two epidermal growth factor (EGF)-like domains and a large catalytic domain(4). It undergoes several post-translational modifications before its secretion by the liver including γ-carboxylation of 10 glutamic acid residues in the Gla domain, N-glycosylation of residues Asn^{145} and Asn^{322}(5) and O-glycosylation of residues Ser^{52} and Ser^{60} in the first EGF domain(6). Hereditary FVII deficiency is a rare autosomal recessive bleeding disorder(7,8). Patients with FVII deficiency have been classified with respect to the plasma level of FVII antigen (VII:Ag) or crossreacting material (CRM) as either CRM^- (low or absent antigen), CRM^R (reduced antigen), or CRM^+ (normal antigen). More than 30 different naturally occurring mutations in the FVII gene have been reported(9). Most are point mutations that alter FVII function, but others interfere with FVII biosynthesis. In this paper, we investigated the mechanisms responsible for FVII deficiency in two homozygous Italian patients who were CRM^- and CRM^R as a result of mutations in the molecule's second EGF domain.

Materials and methods

Collection and processing of blood samples. Blood was collected by atraumatic venipuncture into plastic tubes containing 1/10th volume 0.129 mol/L buffered trisodium citrate.

Plasma was obtained by centrifugation at

2,500g for 15 minutes at 4°C, transferred into plastic tubes, and stored along with the cellular elements at -80°C until use.

FVII assays. FVII coagulant activity (VII:C) and VII:Ag were measured by one-stage clotting assay using recombinant human tissue factor (RecombiPlastin, Ortho Diagnostic Systems, Raritan, NJ) and an enzyme-linked immunoabsorbent assay (ELISA) (American Bioproducts Co, Parsippany, NJ), respectively. A normal pool was constructed by mixing equal volumes of plasma from 30 healthy control subjects.

DNA isolation and in vitro amplification using polymerase chain reaction (PCR). DNA was purified by standard techniques from leukocyte nuclei obtained from whole blood(10). Oligonucleotides and PCR conditions used to amplify the entire coding sequence of the FVII gene have been described in detail(11). PCR amplifications (12) were performed using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). PCR products were generated in 20 μL reaction mixtures that contained 200 ng of genomic DNA, 0.4U of Taq DNA polymerase (Perkin Elmer Cetus), oligonucleotide primers at a concentration of 0.5 μmol/L each, dNTPs at a concentration of 100 μmol/L each,1 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, and 0.01 mg/mL of autoclaved gelatin.

Cloning and sequencing of PCR fragments. Amplified PCR fragments were purified and ligated into PT7Blue T-vectors (Novagen, Madison, WI). Cloned inserts were sequenced by the dideoxy chain termination method on an Applied Biosystems 373A DNA Sequencer (Foster City, CA). All sequence alterations were confirmed in at least two independent clones. Sequence analyses were performed using the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc, Madison, WI) from the Molecular Biology Computer Research Resource (Boston University, Boston, MA).

Restriction enzyme analysis. Restriction enzyme digestion of PCR fragments was used to detect mutations that introduced or abolished a restriction site. Ten μL of PCR-amplified product were digested with 0.5 U of enzyme in a final volume of 20 μL for 16 hours. Restriction fragments were subjected to electrophoresis in nondenaturing 8% (wt/vol) polyacrylamide gels. After completion of the electrophoretic procedure, gels were stained in 0.5 μg/mL ethidium bromide for 5 minutes and photographed under ultraviolet (UV) transillumination.

Antibodies. The production and purification of Mab1476,(13) a murine monoclonal antibody (MoAb), which recognizes an epitope in the aminoterminal region of FVII, has been described(14). This antibody was used to detect FVII in pulse-chase experiments and for immunohistochemistry.

Construction of expression vectors and site-directed mutagenesis. A 2.4 Kb complete human FVII complementary DNA (cDNA)(4) with *Eco*RI/*Bam*HI linkers at

each end was provided by Dr Earl W. Davie (Seattle, WA).

This cDNA was cloned into the *Eco*RI site of the PT7-BlueT vector to obtain the plasmid $PT7^{EcoRI}FVIIwt^{EcoRI}$ and subsequently modified as previously reported to obtain $PT7^{SalI}FVIIwt^{EcoRI}$(14). The FVII cDNA was then isolated and cloned into pED-mtx^r provided by Dr Randal J. Kaufman (15) to obtain the plasmid pEDFVIIwt, a dicistronic messenger RNA mammalian expression vector carrying the dihydrofolate reductase (DHFR) gene at the 3' open reading frame. To investigate the influence of the $Gly^{97}Cys$ and $Gln^{100}Arg$ substitutions on FVII levels, $pEDFVIICys^{97}$ and $pEDFVIIArg^{100}$ were obtained by site-directed mutagenesis of $PT7^{SalI}FVIIwt^{EcoRI}$ using a commercially available kit (Transformer Site-Directed Mutagenesis Kit, Clontech, Palo Alto, CA). Oligonucleotides(5'-GAACGGCTGCTG TGAGCAGTACTGCAGTGATCACACG-3') and (5'-GAACGGCGGCTGTGAGCGTAC TGCAGTGATCACACG-3') spanning nucleotides 7817 to 7853 of the human FVII cDNA were used to introduce a G to T at position 7824 and an A to G at position 7834 (bold letters) coding for $Gly^{97}Cys$ or $Gln^{100}Arg$, respectively. These primers also introduced a *Bcl*I restriction site (underlined), arising from a silent GAC to GAT mutation at nucleotide 7847, to facilitate screening for clones carrying the mutations. The mutant FVII cDNA were then inserted into the pED expression vector to obtain $pEDFVIICys^{97}$ and $pEDFVIIArg^{100}$.

Several restriction enzyme digests were performed to confirm that we had produced vectors containing the mutations. A *Sal* I-*Eco*RI digest released a 2.4 kb fragment containing the complete FVII cDNA and 1.2 kb of the gene's 3' untranslated region, thereby confirming that the entire cDNA had been introduced into the vector. A *Hin*dIII digest of the vector generated three fragments of 955, 2735, and 4000 bp resulting from three sites in pED and none in the FVII cDNA. *Eco*RI digestion linearized the 7.7 kb construct (5.3 kb pED+ insert 2.4 kb cDNA) by cleavage at a single site in pED. To confirm the presence of the mutations, we amplified from the construct a 893 bp fragment with the oligonucleotides 5'-CCCG**GTCGAC**TCAACAGG CAGGGGCAGCACT-3' (position -94 to -74) introducing a *Sal* I site (bold letters) and 5'-CAGGCGGAGCAGCG-3' (position 10648-10661 in exon 8). This PCR product coded for the first 248 amino acids of FVII excluding exon 1b. Because the mutagenic primers introduced a *Bcl* I site in addition to the mutation, a *Bcl* I digest of the product gave two fragments of 695 and 198 bp for the FVIIwt construct, whereas the $FVIICys^{97}$ and $FVIIArg^{100}$ constructs yielded fragments of 458, 237, and 198 bp confirming that the mutations had been introduced. Moreover, because the G to T transversion at position 7824 resulting in $Gly^{97}Cys$ introduces a *Bbv* I site, *Bbv* I digestion of the 893 bp fragment from $pEDFVIICys^{97}$ led to the generation of a 713 bp product that was cleaved into pie-

ces of 445 and 268 bp in the presence of the mutation. As the A to G transversion at position 7834 resulting in $Gln^{100}Arg$ introduces a ^{Bsr}BI site, ^{Bsr}BI digestion of the 893 bp product from $pEDFVIIArg^{100}$ generates fragments of 447 and 446 bp fragments in the presence of the mutation.

Cell culture and transfection assays. For transient transfection experiments, Monkey COS-1 cells (ATCC CRL1650; American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mmol/L L-glutamine, 10 mmol/L HEPES pH 7.2, 100 units/mL of penicillin G, 100 μg/mL streptomycin, and 5 μg/mL of vitamin K1 (AquaMEPHYTON, Merck & Co Inc, West Point, PA) in a 5% CO_2 atmosphere at 37°C. Twenty hours before transfection, COS-1 cells were plated on 60 mm culture dishes at a density of 1 x 106 cells/dish. Five micrograms of the pEDFVII constructs was transfected into cells by Lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturers' instructions. After 16 hours, medium was changed, and 36 hours later, supernatants and cell lysates were harvested and assayed for VII:C and VII:Ag. Results of transient assays are expressed as the percentage of FVIIwt and represent the mean ± SE.

To obtain stable cell lines expressing recombinant FVIIwt, $FVIICys^{97}$, and $FVIIArg^{100}$, we used DHFR-deficient Chinese ovary (CHO) cells (CHO-DUKX-B11)(16) provided by Dr Barbara C. Furie (Boston, MA). These cells were grown in alpha-modified essential medium (AMEM) supplemented with 10% FBS, 2 mmol/L L-glutamine, 10 mmol/L HEPES pH 7.2, 100 units/mL of penicillin G, 100 μg/mL streptomycin, 5 μg/mL of vitamin K1, 10 μg/mL adenosine, 10 μg/mL deoxy-adenosine, and 10 μg/mL thymidine. CHO-DUKX-B11 cells were plated on 100-mm culture dishes at a density of 3 x 106 cells/dish. Transfections were performed as described above with 20 μg of plasmid and 45 μL of Lipofectamine. Two days after transfection, cells were divided at a 1 to 8 ratio and selected for DHFR expression using medium deficient in ribonucleosides and deoxyribonucleosides. Twelve days after transfection, 24 colonies were picked at random and isolated in 12-well (24 mm) plates. At day 20, when the cells achieved confluence, each well was split into two 35 mm dishes. Two days later, cell lysates of the wells were harvested and assayed for VII:Ag. A single clone stably transfected with each construct and expressing high levels of FVII was selected for further experiments. The rates of intracellular FVII synthesis for the selected clones expressing FVIIwt, $FVIICys^{97}$, and $FVIIArg^{100}$ were 33.3 ± 2.1, 52.5 ± 5.8, and 47.7 ± 5.5 ng/mL/h (mean ± SEM, n = 9), respectively.

Metabolic labeling studies. Nearly confluent 60 mm dishes of CHO cells stably expressing recombinant FVII were used

for pulse-chase experiments. Fresh media with FBS was added 4 hours before cells were deprived of methionine for 45 minutes and labeled for 15 minutes with 0.4 mL of methionine-free AMEM (GIBCO-BRL, Gaithersburg, MD) containing 110 μCi Expre^{35}S Protein Labeling Mix (~73% L-[^{35}S] methionine and ~22% L-[^{35}S] cysteine; DuPont NEN Research Products, Billerica, MA) in a 5% CO_2 atmosphere at 37°C. A chase was then performed in 1 mL medium containing an excess of unlabeled L-methionine (GIBCO-BRL, Gaithersburg, MD) for various time periods. At each time point, medium was harvested and phenylmethyl sulfonyl fluoride (PMSF) added to a final concentration of 1 mmol/L. Cell extracts were prepared in 350 μL ice-cold NP-40 lysis buffer (50 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1% (wt/vol) NP-40) supplemented with 1 mmol/L PMSF. The cell lysates were precleared overnight at 4°C with 50 μL of 20% (vol/vol) fixed *Staphylococcus aureus*. Cowan I (SAC) coupled with a rabbit antimouse IgG (Sigma, St Louis, MO) in NP-40 lysis buffer. Immunoprecipitation of FVII was accomplished by incubating precleared cell lysates and conditioned media with 4 μg of MoAb MC1476 for 2 hours at 4°C. The resulting immune complexes were adsorbed with 30 μL of 20% (vol/vol) Protein A Sepharose FF (Sigma) coupled 5:1 (vol/vol) with rabbit antimouse IgG antiserum in NP-40 lysis buffer. Pellets were washed four times in NP-40 lysis buffer and resuspended either in buffer for further enzymatic digestion (see below) or in polyacrylamide gel electrophoresis (PAGE) sample buffer with or without reducing agents, and denatured by heating to 95°C for 5 minutes. The immunoprecipitated proteins were resolved by sodium dodecyl sulfate (SDS)-PAGE in 8% (wt/vol) gels, and analyzed by fluorography on X-OMAT-AR film (Eastman-Kodak Co, Rochester, NY) after treatment with En^{3}Hance (DuPont NEN Research Products, Billerica, MA). To quantitate the relative amount of FVII immunoprecipitated at each time, the radioactivity incorporated into bands containing FVII was analyzed with the Umax PowerlookII Imaging Analyzer (Umax Data System Inc, Taiwan).

Immunohistochemistry. CHO cell lines expressing FVII were grown overnight at 150,000 cells/glass coverslip (Baxter Scientific products, McGawpark, IL). The coverslips were washed once in phosphate-buffered saline (PBS) and fixed for 1 hour in 3% (vol/vol) paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS. The cells were sequentially washed, permeabilized for 3 minutes in 0.1% (vol/vol) Triton X-100 (Sigma), and washed three more times. They were then incubated in 0.15% (wt/vol) glycine containing 0.1% (wt/vol) bovine serum albumin (BSA) for 15 minutes followed by anti-FVII Mab (4 μg in 0.5 mL of PBS containing 1% BSA) for 30 minutes. The cells were again washed three times and then incubated with fluorescein isothiocyanate (FITC)-labeled goat-antimouse IgG (1/1000 in PBS containing

1% BSA) (Cappel, Durham, NC) for 30 minutes. After further washing, the coverslips were mounted on glass slides with Airvol (Air Products, Allentown, PA). Immunofluorescence microscopy was performed on a Zeiss axioplan fluorescence microscope at 630 x magnification (Zeiss, Thornwood, NY).

Effect of protein degradation inhibitors on FVIIwt, FVIICys97, and FVIIArg100 levels. To study the effect of various protein degradation inhibitors on FVII biosynthesis, confluent stably transfected CHO cells grown in 60 mm dishes were incubated with media containing either lactacystin (10 μmol/L) (Calbiochem, La Jolla, CA), ammonium chloride (50 mmol/L), leupeptin (100 μmol/L), N-acetyl-Leu-Leu-Norleucinal (50 μg/mL), or brefeldin A (10 μg/mL) (Sigma) dissolved according to the manufacturers' recommendations and used at previously published concentrations(17-21). Four hours later, cell lysates were harvested and assayed for VII:Ag.

Analysis of N-linked glycosylation and sialation. Immunoprecipitated proteins were incubated with 100 mU/mL Endo-β-N-acetylglucosaminidase H (Endo H) or 9.4 U/mL Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase (N-glycanase) for 18 hours at 37° C according to the manufacturer's instructions. For sialation analysis, immunoprecipitated proteins were incubated for 1 hour at 37°C with 4 U/mL neuraminidase in 1 mmol/L PMSF, 20 mmol/L Tris-maleate pH 6.0, 10 mmol/L calcium acetate, and 1.75% (vol/vol) NP-40. All enzymes were from Genzyme (Cambridge, MA). The reactions were terminated by adding 5x PAGE sample buffer, and were analyzed by SDS-PAGE.

Informed consent. Informed consent to perform research studies was obtained from the patients. The study was approved by the Human Studies Committee of the Brockton-West Roxbury VA Affairs Medical Center.

Results

Patients and genomic studies. We investigated the molecular defects in two Italian patients with FVII deficiency. Patient 1 is a 28-year-old male with a mild hemorrhagic diathesis manifested by epistaxis and excessive bleeding after dental extractions (22). Patient 2 is a 53-year-old woman with severe bleeding evidenced by recurrent hemarthrosis with chronic arthropathy and an episode of cerebral hemorrhage(22). The levels of VII:Ag and VII:C in the two patients were 2% and less than 1% of normal and 12% and less than 1% of normal, respectively (Table 1).

For each patient, we subcloned and sequenced the entire coding region and the exon/intron boundaries of the FVII gene as well as its 5'-flanking region. In patient 1, a G to T transition at position 7824 (GGC to TGC) and a C to T transition at position 7880 (CAC to CAT) in exon 5 were identified resulting in $Gly^{97}Cys$ and a neutral dimorphism in the codon for His^{115}, respectively. Two previously described

Tab. 1 - **Coagulation data and genetic alterations in two italian patients with FVII deficiency**

Patient	VII:Ag (%)	VII:C (%)	Gly97Cys	Gln100Arg	Arg353Gln	-323 Insert
1	2	<1	+/+	-/-	+/+	+/+
2	12	<1	-/-	+/+	-/-	-/-

Presence or absence of the sequence alteration in each allele is denoted by + or - signs, respectively.

FVII polymorphisms known to influence FVII levels, a C to A substitution at position 10976 (CGG to CAG) in exon 8 resulting in $Arg^{353}Gln$ and the insertion of a decanucleotide at position -323 in the 5'-flanking region of the FVII gene, were also present. In patient 2, an A to G transversion at position 7834 (CAG to CGG) in exon 5 resulting in $Gln^{100}Arg$ was identified. No alleles with wild-type sequence were identified in either patient. To definitively establish that the patients were homozygous, we tested for the presence of the various sequence alterations using restriction enzyme analysis. The G to T transversion at position 7824, resulting in $Gly^{97}Cys$, introduces a single *Bbv* I site. Digestion of a 313 bp PCR product spanning exon 5 with *Bbv* I generates fragments of 170 and 143 bp in the presence of the mutation. The A to G transversion at position 7834, resulting in $Gln^{100}Arg$, introduces a *Bsr*BI site and digestion with this enzyme generates fragments of 147 and 166 bp in the presence of the mutation. *Msp* I restriction analysis was used to identify the $Arg^{353}Gln$ polymorphism(23), whereas the decanucleotide insert at position -323 was assessed by visualization of a 10 bp difference in fragment size after *Eco*RI digestion of a PCR fragment spanning this region(24).

Based on the results of these restriction analyses (data not shown), pa-tient 1 was ho-mozygous for $Gly^{97}Cys$ and the two polymorphisms whereas patient 2 was homozygous for only $Gln^{100}Arg$ (Table 1).

Transient transfection assays in COS-1 cells. To investigate the influence of the $Gly^{97}Cys$ and $Gln^{100}Arg$ substitutions on FVII biosynthesis, transient transfections were performed in COS-1 cells using the dicistronic pED vectors containing either wild-type or mutant FVII cDNAs. Assays of VII:Ag in cell lysates showed that $FVIICys^{97}$ and $FVIIArg^{100}$ were reduced to 38% and 54% of FVIIwt, respectively, whereas the levels in the conditioned media were decreased to 6.6% and 16.7% of FVIIwt, respectively (n=8). The small amount of $FVIICys^{97}$ released into the media was functionally active as assessed by VII:C assay (6.1% ± 0.3% of wt) because the level was similar to VII:Ag (6.6% ± 0.5%). In contrast, the $Gln^{100}Arg$ mutation impaired the molecule's function because the VII:C level in the media (2.8% ± 0.2%) was considerably lower than VII:Ag (16.7% ± 2.1%) (Table 2).

Expression studies in stably transfected cell lines. Based on the results of the transient transfection experiments, it

Tab. 2 - **Transient expression assays of pEDFVIIwt, pEDFVIICys^{97}, and pEDFVIIArg^{100} in COS-1 Cells**

	FVIIwt	FVIICys97	FVIIArg100
Cell lysate, VII:Ag	100 ± 2.8	38.1 ± 3.9	54.6 ± 3
Conditioned media, VII:Ag	100 ± 3	6.6 ± 0.5	16.7 ± 2.1
Conditioned media, VII:C	100 ± 2.9	6.1 ± 0.3	2.8 ± 0.2

FVII levels were measured in cell lysates and conditioned media 36 hours after 8 independent transfections of cells from a single clone transfected with each construct. Results are expressed as the percentage of FVII (mean ± SE) produced by the wild-type construct.

appeared that the Gly^{97}Cys and Gln^{100}Arg mutations led to impaired FVII biosynthesis. To study these defects, pEDFVIIwt, pEDFVIICys^{97}, and pEDFVIIArg^{100} were transfected into DHFR-deficient CHO cells to obtain stably transfected cell lines. It can be observed that the amounts of FVII in cell lysates of the stable cell lines transfected with pEDFVIICys^{97} and pEDFVIIArg^{100} were actually greater than FVIIwt. This is attributable to the selection of high-level FVII producers. After a 15 minute pulse with [^{35}S] methionine, the recombinant FVIIwt in cell lysates was maximal at 30 to 60 minutes and decreased as the protein was secreted (Fig 1, top). FVIICys^{97} was synthesized at a rate similar to FVIIwt with maximal accumulation at 60 minutes, but remained in the cell for a longer period of time. Approximately, 40% of the maximal amount of intracellular FVIICys^{97} was still present at 240 minutes as compared with 12% for FVIIwt (Fig 1, top). FVIIArg^{100} was retained intracellularly even longer with maximal accumulation at 60 to 120 minutes, and 55% of the protein was retained in the cell at 240 minutes. In the conditioned media, FVIIwt was barely detectable at 30 minutes and then proceeded to accumulate (Fig 1, bottom). The levels of FVIICys^{97} and FVIIArg^{100} were much lower than that of FVIIwt at all time points. At 240 minutes, the levels of FVIICys^{97} and FVIIArg^{100} in the media were 30% and 16% of FVIIwt, respectively (Fig 1, bottom).

Using immunohistochemical techniques to detect intracellular VII:Ag, we found different patterns of localization for FVIIwt, FVIICys^{97}, and FVIIArg^{100}. Staining of FVIIwt (Fig 2A and B) and FVIICys^{97} (Fig 2C) was mostly perinuclear suggesting that these molecules were localized primarily in the Golgi apparatus. In contrast, FVIIArg^{100} (Fig 2D) staining was predominantly diffuse without perinuclear enhancement, suggesting that it was retained for a longer duration of time in the endoplasmic reticulum (ER) than the other recombinant proteins.

Whereas the acquisition of Endo H resistance is frequently employed to monitor protein transit to the Golgi complex from the ER, the radioactive bands that were observed for FVIICys^{97} and FVIIArg^{100} in pulse-chase experiments were generally of low intensity that precluded interpretation of these experiments (data not shown).

Effects of protein degradation inhibitors on FVII biosynthesis. We next

analyzed the effects of various inhibitors of protein degradation on intracellular FVII levels in stably transfected cells expressing FVIIwt, FVIICys^{97}, and FVIIArg^{100}. Inhibitors of lysosomal degradation, including NH_4Cl, which inactivates lysosomal enzymes by pH modification, and leupeptin, which inhibits cathepsins B, D, H, and L, did not increase intracellular FVII levels (Table 3) suggesting that FVIICys^{97} and FVIIArg^{100} were not degraded in the lysosome. Lactacystin, a potent specific inhibitor of proteasome degradation, also did not increase FVII levels (Table 3). However, ALLN, a neutral inhibitor of the cysteine proteases, calpain, and cathepsin D, induced a significant increase in FVIICys^{97} levels (147% with ALLN versus 100% without ALLN, P = .0004) without altering the levels of FVIIwt or FVIIArg^{100}. This data therefore suggested that FVIICys^{97} is degraded intracellularly.

Tab. 3 - **Effect of various inhibitors of protein degradation and brefeldin A on intracellular levels of FVIIwt, FVIICys^{97}, and FVIIArg^{100}**

	FVIIwt	FVIICys97	FVIIArg100
Media alone (n = 6)	100 ± 1.1	100 ± 3.1	100 ± 4.2
NH_4Cl, 50 mmol/L (n = 3)	107 ± 4.2	83.4 ± 3.6	113 ± 2.7
Leupeptin, 100 µmol/L (n = 3)	98 ± 1.2	100.5 ± 3.9	84 ± 3.6
Lactacystin, 10 µmol/L (n = 3)	88 ± 1.6	91.4 ± 2.8	98.1 ± 5.5
ALNN, 50 µg/mL (n = 9)	96.4 ± 5.7	147 ± 8.9*	113 ± 4.1
Brefeldin A, 10 µg/mL (n = 6)	190 ± 10.4°	125 ± 7.5#	151 ± 10.8§

90% confluent stably transfected CHO cells expressing recombinant FVIIwt, FVIICys^{97}, or FVIIArg^{100} were incubated 4 hours in fresh media containing 10% FBS in the presence or absence of the different agents. The figures in parentheses are the number of independent transfections performed on cells from a single clone expressing each construct. The levels of VII:Ag were then measured in cell lysates and the results are expressed as the percentage of FVII (mean ± SE) produced in the absence of the agent.

** P = .0004, ° P = .0001, #P = .02, § P = .002*

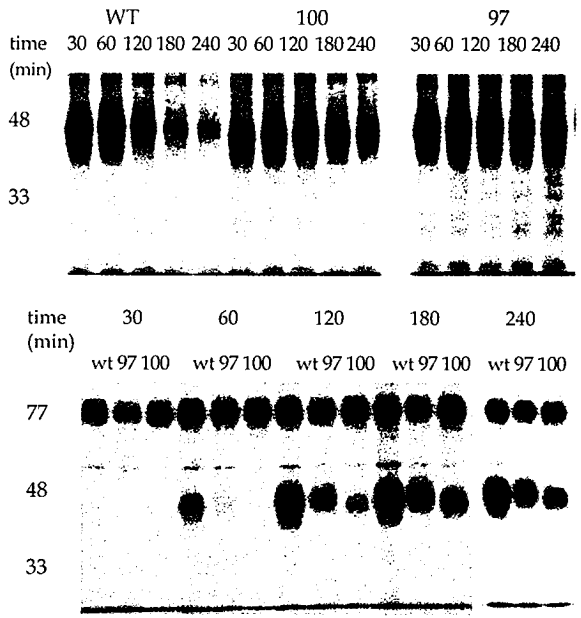
To localize the site of degradation, we investigated the effect of brefeldin A on intracellular levels of FVII in the three cell lines. Brefeldin A blocks protein transport from the ER to the Golgi complex and causes translocation of Golgi components back to the ER. After incubation with brefeldin A, FVIIwt and FVIIArg^{100} increased significantly within the cells to 190% and 151% of control levels, respectively, consistent with normal translocation of these two proteins from the ER to the Golgi in the absence of the drug. In contrast, the intracellular level of FVIICys^{97} increased only modestly in the presence of brefeldin A to 125% of control levels, thereby suggesting that the degradation of this molecule occurred primarily in a pre-Golgi compartment. Analysis of immunoprecipitates obtained after a 45 minute pulse with 110 µCi [^{35}S] methionine and 2 hours of chase in the presence and absence of 10 µg/mL of brefeldin A confirmed that FVIIwt and FVIIArg^{100} levels in cell lysates increased to 188% and 180% of control levels respectively as measured by imaging analysis of radioactive bands, whereas FVIICys^{97}

levels were unaffected (105% versus 100% in control cells) (data not shown).

Analysis of altered mobility of FVIIArg^{100}. In Fig 1, FVIIwt and FVIICys^{97} from conditioned media are visualized by nondenaturing PAGE as a single band with an apparent mol wt of 48 kD. This is slightly greater than that for the intracellular FVII species and results from post-transcriptional modifications of the protein. However, FVIIArg^{100} from the conditioned media appeared as a single band with a faster electrophoretic mobility than FVIIwt on nonreducing 8% SDS-PAGE. A smaller difference in electrophoretic mobility between FVIIwt and FVIIArg^{100} was also observed in the cell lysates. We hypothesized that this difference in electrophoretic mobility resulted from either a change in glycosylation or alteration of the molecule's secondary structure.

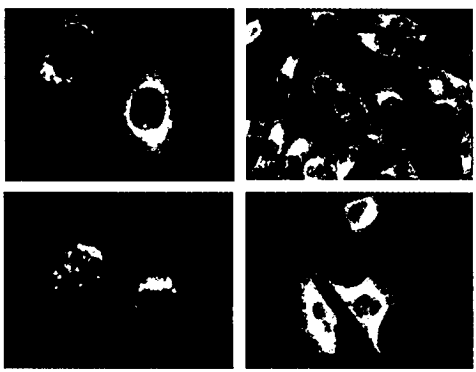
To investigate N-linked glycosylation, radiolabeled FVII was immunoprecipitated and incubated with Endo-H or N-Glycanase which respectively cleaves either high-mannose and certain hybrid-type carbohydrates at the GlcNAcβ1-4GlcNAc linkage to leave a single GlcNAc residue attached to asparagine (25) or all N-linked carbohydrates by hydrolyzing the asparginyl-oligosaccharide bond. (26) As the secretion of FVIIwt and FVIIArg^{100} occur at different rates, we evaluated lysates from the two stably transfected cell lines at different chase times. After a 15-minute pulse with [^{35}S] methionine and 120 minutes of chase time, FVIIwt migrated more rapidly after digestion with either Endo H or N-Glycanase, reflecting the removal of N-linked high mannose oligosaccharides from the

Fig. 1 - **Pulse-chase labeling experiments in CHO cells transfected with pEDFVIIwt, pEDFVIICys^{97}, and pEDFVIIArg^{100}.**



After a 15-minute pulse with [^{35}S] methionine, the cells were chased for 30, 60,120, 180, and 240 minutes. Equivalent amounts of cell lysate (top) or conditioned media (bottom) for FVIIwt (wt), FVIICys^{97} (97) and FVIIArg^{100} (100) were immunoprecipitated using a Mab against FVII and analyzed by 8% SDS-PAGE under nonreducing conditions. The location of molecular weight markers in kD is denoted at the left-hand side of the figure. In the conditioned media (bottom), the 77 kD band was nonspecific as it was observed with equal intensity in untransfected CHO cells (data not shown).

Fig. 2 - **Immunohistochemical localization of wild-type and mutant FVII molecules in stably transfected CHO cells.**



FVIIwt (A, B) and FVIICys ^{97} (C) were mostly localized in the perinuclear area, whereas FVIIArg^{100} (D) was present diffusely throughout the cytoplasm without perinuclear enhancement. Untransfected CHO cells did not react with either the anti-FVII Mab or the fluorescent second antibody indicating that the observed labeling was specific for FVII (data not shown).

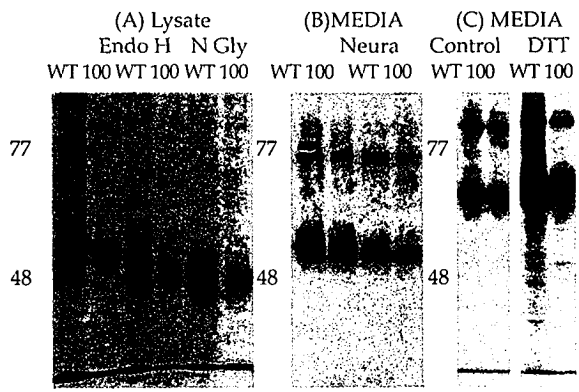
nascent 46 kD protein (Fig 3A).

FVIIArg^{100} behaved similarly to FVIIwt at 180 minutes, suggesting that N-linked glycosylation of this molecule was occurring normally. To detect the presence of sialic acid on the glycans of FVIIwt and FVIIArg^{100}, we performed digestion with neuraminidase. Recombinant FVIIwt and FVIIArg^{100} both migrated more rapidly after digestion with neuraminidase, but the difference in electrophoretic mobility of FVIIArg^{100} as compared with FVIIwt remained (Fig 3B). This suggested that modification of the glycans with sialic acid was similar for the two molecules. However, after reduction of the disulfide linkages with 100 mmol/L dithiothreitol, FVIIwt and FVIIArg^{100} migrated with a similar electrophoretic mobility (Fig 3C), thereby suggesting that disulfide bond formation of the FVII molecule was affected by the Gln^{100} Arg substitution. Similar results were obtained after reduction with 5% (vol/vol) β-Mercaptoethanol (data not shown).

Discussion

We investigated the mechanisms responsible for FVII deficiency in two unrelated Italian patients with a bleeding diathesis. Sequencing of the coding sequences and intron/exon boundaries of the FVII gene showed that they were homozygous for different mutations within the coding region for exon 5. Patient 1 had a G to T mutation at position 7824 and patient 2 had an A to G mutation at position 7834 resulting in Gly^{97}Cys and Gln^{100}Arg, respectively. These two mutations were previously reported in association with other mutations in doubly heterozygous patients.(27-29)Patient 1, who was homozygous for Gly^{97}Cys, was also homozygous for two polymorphisms known to reduce FVII levels, Arg^{353}Gln in exon 8 and the decanucleotide insert in the 5' flanking region of the FVII gene. (23,30,31) To study the mechanism by which the two mutations reduce FVII levels, we performed transient expression studies in COS-1 cells with cDNA encoding FVIIwt, FVIICys^{97}, and FVIIArg^{100} and showed that the secretion of the two mutant proteins

Fig. 2 - **Analysis of the altered electrophoretic mobility of FVIIArg^{100}.**



After a 15-minute pulse with [^{35}S] methionine, stably transfected cells expressing FVIIwt and FVIIArg100 were chased for 60 minutes and 120 minutes, respectively, to analyze FVII in cell lysates. In the conditioned media, FVIIwt and FVIIArg100 were both investigated after 180 minutes of chase. FVIIwt (wt) and FVIIArg100 (100) were immunoprecipitated using a MoAb against FVII. (A) Equivalent amounts of cell lysate were analyzed by 8% SDS-PAGE before (-) or after treatment with Endo H and N-Glycanase (N-Gly). (B) Conditioned media were analyzed before (-) or after treatment with neuraminidase (Neura) by 10% SDS-PAGE. (C) Conditioned media were evaluated under nonreducing conditions (control) or after reduction by 100 mmol/L Dithiothreitol (DTT). The 77 kD band was nonspecific because it was also observed in conditioned media from untransfected CHO cells (data not shown). Molecular weight markers in kD are indicated on the left.

was impaired. The levels of VII:Ag and VII:C in the conditioned media were reduced concordantly for FVIICys^{97}, whereas a higher level of VII:Ag relative to VII:C was observed for FVIIArg^{100}. The results were similar to those in the patients' plasmas. We also observed that the intracellular levels of VII:Ag were reduced in transient transfection assays performed with the mutant cDNA.

To study the intracellular processing of the mutant proteins, we performed pulse-chase experiments and immunohistochemical staining of stably transfected CHO cells expressing FVIICys^{97} and FVIIArg^{100} in comparison to FVIIwt. Our results showed that the proteins did not accumulate intracellularly in spite of major secretion defects. A potential mechanism to account for reduced amounts of FVII protein in the cells is degradation. Intracellular degradation of abnormal proteins can result from lysosomal proteolysis or from pre-Golgi or ER degradation, which has been termed the "quality control function" of the ER(32,33). Protein degradation within the ER is a complex, poorly understood process and occurs either within the ER lumen or on the cytoplasmic side. Some of the proteolytic events are adenosine triphosphate (ATP)-dependent and several of the enzymes involved in this process are sensitive to serine protease inhibitors. Some proteins with an abnormal conformation are degraded by a soluble ATP-dependent pathway in which conjugation occurs between the abnormal protein and multiple molecules of ubiquitin followed by hydrolysis by a 26S proteolytic enzyme complex. The proteolytic core of this structure is the 20S proteasome, which has been localized to the cytoplasmic surface of the ER by immunoelectron microscopy (34).

To investigate potential mechanisms of intracellular degradation, we examined the biosynthesis of FVIICys^{97} using inhibitors of lysosomal pH (NH_4Cl), lysosomal proteolytic enzymes (leupeptin), and the 20S-proteasome (lactacystin). We also evaluated the effect of ALLN, a common inhibitor of neutral Ca^{2+}-dependent cysteine proteases, which inhibits the ubiquitin-proteasome pathway (35). The intracellular level of FVIICys^{97} did not change in the presence of inhibitors of lysosomal function (ie, NH_4Cl and leupeptin) or lactacystin, whereas a significant increase was observed with ALLN. These data indicate that FVIICys^{97} degradation does not occur in lysosomes and involves a cysteine protease, which is, however, independent of the proteasome pathway.

Brefeldin A inhibits protein secretion in a pre-Golgi compartment such that proteins destined for secretion remain in the ER(36). Whereas the level of FVIIwt rose by 90% of baseline in the presence of brefeldin A, the intracellular level of FVIICys^{97} increased by less than 30%. The Gly^{97}Cys mutation is in the second EGF domain of FVII and results in substitution of a small nonpolar side chain by a polar side chain. Based on the crystal structure of activated FVII bound to the extracellular domain of tissue factor(37), Gly^{97} helps position the Cys^{98}-Cys^{112} loop to facilitate intramolecular disulfide bonding. We postulate that the resulting alteration in protein conformation causes FVIICys^{97} to undergo degradation in a pre-Golgi compartment.

For FVIIArg^{100}, pulse-chase experiments showed that the mutant protein was retained for a longer time interval in stably transfected CHO cells than FVIIwt and the level was not increased by the various inhibitors of protein degradation. In the presence of brefeldin A, the levels of FVIIArg^{100} increased by 51% as compared to FVIIwt that increased by 90%. This data coupled with the immunohistochemical demonstration that FVIIArg^{100} is present diffusely throughout the cytoplasm is consistent with retention of a substantial portion of the mutant protein in the ER.

The more rapid electrophoretic mobility of FVIIArg^{100} as compared with FVIIwt suggested that FVIIArg^{100} might be incompletely glycosylated or have an abnormal tertiary structure. To investigate alterations in N-linked glycosylation and sialation, radiolabelled FVIIwt and FVIIArg^{100} from conditioned media were treated with Endo H, N-Glycanase, and neuraminidase. The difference in electrophoretic mobility remained between FVIIwt and FVIIArg^{100} after digestion with the three enzymes, implying that N-linked glycosylation and sialation of FVIIArg^{100} were unaffected. We did not evaluate whether O-linked sugars on residues Ser^{52} and Ser^{60} of FVII (6,38) were altered because FVIIAla^{52}, a mutant molecule lacking glycosylation at this residue, has been reported to have the same electrophoretic mobility as FVIIwt(38). Because the second EGF domain contains three intramolecular disulfide bonds (Cys^{91}-Cys^{102}, Cys^{98}-Cys^{112}, and Cys^{114}-Cys^{127}),

we compared the electrophoretic mobility of FVIIwt and FVIIArg^{100} under reducing as well as non-reducing conditions. After reduction, the difference in electrophoretic mobility between the two molecules was no longer present, leading us to speculate that FVIIArg^{100} does not undergo normal disulfide bonding during its biosynthesis. To confirm this, chemical determination of the disulfide bonding pattern of FVIIArg^{100} would need to be performed and the results compared with those for FVIIwt. The mutation replaces a neutral Gln residue with a larger positively-charged Arg, which may sterically interfere with Tyr^{118} and electrostatically interfere with the positively-charged side chain of His^{115}(37). We therefore postulate that the Gln^{100}Arg mutation in FVII results in a protein with an abnormal conformation that remains in the ER for an extended period during its biosynthesis and reduces its secretion.

Whereas defective biosynthesis is a major defect conferred by the Gln^{100}Arg mutation, a small amount of dysfunctional protein is secreted as evidenced by the low specific activity of the FVII (ie, ratio of VII:C to VII:Ag) that is present in the patient's plasma as well as conditioned medium of transfected COS-1 cells. Our data is in agreement with studies of FVIIArg^{100} reported by Kemball-Cook *et al*(39), who showed that the activated form of the mutant recombinant protein had markedly diminished affinity for tissue factor and, in complex with soluble tissue factor, had less than 5% of the ability of FVIIwt to activate factor X. Orning *et al* (40) have also provided data that the peptide sequence, Glu^{99}-Gln^{100}-Tyr^{101}, in the second EGF domain of FVII, inhibits the ability of FVIIwt to mediate tissue factor-dependent factor X activation. Based on the crystal structure of the FVIIa-tissue factor complex, Gln^{100} is located at the interface of the second EGF and the protease domain, but is not in contact with tissue factor.

Whereas Gly^{97}Cys and Gln^{100}Arg have previously been reported in double heterozygotes with FVII deficiency, this is the first report of homozygous patients with these mutations. The severe clinical phenotype of patient 2 with Gln^{100}Arg is consistent with studies showing a major secretion defect as well as markedly impaired function of the small amount of FVII that is released from cells. It is unclear why patient 1 with Gly^{97}Cys does not have a similarly severe bleeding diathesis. Because it is not possible to obtain sufficient FVIICys^{97} protein to perform detailed biochemical studies, we cannot evaluate whether a very small amount of circulating FVII protein posseses sufficient biologic function to attenuate the bleeding diathesis.

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Abnormal secretion and function of recombinant human factor VII as the result of modification to a calcium binding site caused by a 15 base pair insertion in the factor VII gene

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Summary

We report a case of a novel insertion type mutation in the factor VII (FVII) gene that results in severe FVII deficiency with FVII coagulant activity (FVII:C) of <1% and factor VII antigen levels (FVII:Ag) of 10%. DNA analysis revealed a homozygous 15 bp in-frame insertion type mutation at nucleotide 10554 within the catalytic domain of FVII. This insertion consists of a duplication of the residues Leu213 to Asp217 (Leu, Ser, Glu, His, Asp), probably arising by slipped mispairing between 2 copies of a direct repeat (GCGAGCACGAC) separated by 4 bp. Molecular graphics analyses of FVIIa showed that the insertion is located at the surface of the catalytic domain in an exposed loop stabilized by extensive salt-bridge and hydrogen bond formation at which the calcium binding site is located. The insertion may interfere with protein folding during FVII biosynthesis and/or diminish functional activity through the loss of calcium binding. To explore these two hypotheses, wildtype FVII (FVIIWT) and mutant FVII (FVIIMT) cDNAs were expressed transiently in COS1 cells and stably in Chinese hamster ovary cells (CHO). In lysates of cells transfected with either the FVIIWT or FVIIMT constructs, the FVII:Ag levels were equivalent. However the amount of FVII:Ag secreted by cells transfected with FVIIMT was 5-10% of that secreted by cells transfected with FVIIWT. Using stably transfected CHO cells, pulse chase studies demonstrated that FVIIMT did not accumulate intracellularly. A part of this recombinant protein was degraded in PreGolgi compartment as demonstrated by using different inhibitors of protein degradation. Accordingly, only small amounts of FVII with not detectable procoagulant activity were secreted into conditioned media. These results verify both the hypotheses derived from inspection of the FVIIa crystal structure, and demonstrate that both a secretion and a functional defect is the mechanism whereby this insertion causes FVII deficiency.

Introduction

Factor VII (FVII) has a central role in the initiation of blood coagulation. This glycoprotein circulates in blood as a single-chain zymogen composed of 406 amino acid residues (1) with a molecular weight of 50 kD. FVII, after the formation of a one-to-one stoichiometric complex with its cell surface receptor and cofactor tissue factor (TF) and in the presence of calcium ions, is rapidly cleaved to its active form, FVIIa. This activation occurs through proteolytic cleavage at a single site (Arg152-Ile153)(2,3). FVIIa converts zymogen factors X and IX to the corresponding active enzymes (2). FVIIa is composed of an N-terminal light chain (152 amino acids) and a C-terminal heavy chain (254 amino acids) linked by a disulfide bond. The light chain contains an amino terminal γ-carboxy-glutamic acid-rich domain, followed by two epidermal growth factor (EGF)-like modules. The heavy chain consists of the catalytic domain. The FVII gene is 12.8 kb in length and is located 2.8 kb away from the factor X gene on the long arm of chromosome

13(4). The mature protein is encoded by exons 2 to 8.

FVII deficiency is a rare recessive bleeding disorder with a relatively poor correlation between FVII coagulant activity and hemorrhagic symptoms(5). Several missense mutations and a few small deletions and insertions in the human FVII gene have been reported(6,7), but no insertion type mutation or mutations affecting the FVII calcium site in the catalytic domain have yet been documented (see the FVII mutation database at http://europium.csc.mrc.ac.uk). Here, using expression studies, we describe and characterize the first case of FVII deficiency caused by a homozygous insertion type mutation consisting of a duplication of a 15 bp segment within exon 8 (Leu213-Asp217) in the catalytic domain ({73-77}: chymotrypsinogen numbering is denoted throughout by curved brackets). Crystal and solution scattering structures are available for FVIIa and its complex with TF(8-11). We show, using molecular graphics analysis, that the insertion is proximate to the calcium binding site of the catalytic domain, and propose that this is responsible for the complete loss of FVIIa activity. *In vitro* transient and stable expression studies demonstrated reduced levels of FVII:Ag in conditioned media, supporting the hypothesis of a secretion defect caused by the insertion mutation. This mutation probably does not interfere with FVII synthesis, but is associated with various defects including abnormal folding, intracellular degradation, secretion failure and loss of proteolytic activity. To our knowledge, this is the first instance of a FVII deficiency caused by a perturbation at its calcium-binding site in the catalytic domain.

Materials and methods

Patient. The patient, born from a consanguineous marriage, is a 5 year old female from Oman. The first bleeding episode was noted after female circumcision. Hematuria occurred in her first year of life and several episodes of epistaxis and hematuria ocurred at age 3. These bleeding episodes were severe enough to require transfusions of fresh frozen plasma and red cells. Informed consent to perform the research studies was obtained from the patient's family.

Collection and processing of blood samples. Blood was collected by atraumatic venepuncture into plastic tubes containing 1/10th volume 0.129 mol/L buffered trisodium citrate. Plasma was obtained by centrifugation at 2500 X g for 15 min at 4°C, transferred into plastic tubes, and stored along with leukocytes at -80°C until use.

Factor VII assays. Plasma FVII:C was measured by a one-stage prothrombin time-based assay, using human recombinant tissue factor (RecombiPlastin, OrthoDiagnostic System, Raritan, NJ) and FVII deficient plasma (Dade International, Miami, FL). Plasma FVII:Ag was measured using an enzyme-linked immunoabsorbent assay (ELISA) using murine monoclonal antibodies against FVII(12). A normal plasma pool was constructed by mixing equal volumes of plasma from 40 healthy subjects, 20 men and 20 women (not pregnant and not taking oral

contraceptives). In the expression studies, levels of recombinant FVII:C and FVII:Ag were determined using the functional assay described above and ELISA (American Bioproducts Co., Parsippany, NJ) using a polyclonal rabbit anti-serum against human FVII, respectively.

DNA isolation and in vitro amplification using PCR. Genomic DNA was isolated from peripheral blood leukocytes by established techniques(13). The coding regions, intron/exon boundaries, and 490 bp of 5' flanking region of the FVII gene were amplified by polymerase chain reaction (PCR) and analyzed for mutations by single-strand conformational polymorphism (SSCP)(14). PCR amplifications were carried out in 100 µl volumes comprised of 100-500 ng of DNA, 100 pmoles of each oligonucleotide primer, 200 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.3, and 2 units of Taq polymerase (Perkin-Elmer Cetus). Reactions were denatured at 94°C for 5 min, then 40 cycles of amplification were performed, with a 10 min extension time in the last cycle. For SSCP analysis, 1 µl of each final PCR product was mixed with 5 µl of denaturation buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromphenol blue), denatured for 10 min at 94°C and then placed on ice for 2 min. 3-4 µl of each denatured sample were loaded onto a 10% polyacrylamide gel (99:1 acrylamide: bisacrylamide) in Tris-borate EDTA buffer (0.09 M Tris-borate, 2 mM EDTA), supplemented with 7.5% urea. Following electrophoresis DNA fragments were visualized by silver staining (Promega Corp., Southampton, UK). PCR fragments showing abnormal SSCP migration were purified by Bioline PCRapid Purification kit (Bioline, London, UK) and directly sequenced using an ABI 377 automated DNA sequencer (Perkin-Elmer Applied Biosciences, Warrington, UK).

Antibodies. The production and purification of Mab1476, a murine monoclonal antibody, which recognizes an epitope in the amino terminal region of FVII, has been described (15). This antibody was used to detect FVII in pulse chase experiments.

Structural analysis of the mutant protein. The crystal coordinates of the TF-FVIIa complex and unbound FVIIa at resolutions of 2.0-2.8 Å (9-11) were obtained from the Protein Data Bank (PDB codes 1dan, 1qfx and 1cvw). Protein structures were visualized using INSIGHT II software (MSI, San Diego, CA) on Silicon Graphics INDY Workstations in conjunction with Crystal Eyes stereo glasses. The FVIIa secondary structures and sidechain solvent accessibilities are as reported previously(7). The rigid body fragment assembly method in the HOMOLOGY and DISCOVER modules (MSI) was used to model the insertion. For reason of their positions in the exposed surface loop, His216 {76} and Asp219 {79} were fixed and used to define the insertion point, and Asp217 {77} and Gly218 {78} were deleted. The seven-residue fragment DLSEHDG was inserted by standard homology modelling methods(16). A precalculated Cα distance matrix identified known loops in the

Tab. 1 - **Effect of inhibitors of protein degradation and Brefeldin A on intracellular levels of FVIIWT and FVIIMT.**

	FVIIWT (%)	FVIIMT (%)
Media alone	95 (± 5)	116 (± 4)
NH_4Cl (50 mM/L)	121 (± 4)	115 (± 3)
Leupeptin (100 µmol/L)	87 (± 18)	99 (± 16)
Brefeldin A (10 µg/mL)	150 (± 3)	88 (± 24)

Confluent CHO cells expressing recombinant FVIIWT and FVIIMT were incubated for 4 h in fresh media containing 10% FBS in the presence or absence of agents. The results report the levels of FVII:Ag in cell lysates, expressed as the percentage of FVII:Ag (mean ± SE) from 3 independent experiments. The p values (see text) were calculated by the t-test.

Protein Data Bank that best fitted the corresponding Cα distance matrix based on the positions of His216 {76} and Asp219 {79} and other flanking residues in FVIIa. Sidechain atoms were automatically generated for the inserted region using template structures and general rules for residue exchanges. The final inserted loop structure showed no steric overlap with the remaining FVIIa structure and was refined using 300 steps of steepest descent energy minimisation.

Cloning of the Insertion Mutation. The 15 bp insertion mutation identified at the very beginning of exon 8 was inserted into the FVIIWT cDNA by overlapping PCR (Figure 1). For the first PCR reaction, the FVIIWT cDNA was used as a template. The upstream primer extended from position 8906 to 8937 in exon 6, including the underlined Xba I restriction site (CCATGTGGAAA AATACCTA TTCTAGAAAAAAG). The downstream primer began at position 9726, the junction of exons 7 and 8 (CCAGCACCGCGACAAATT TCTCCAGTTC) and included silent mutations (underlined) in codons 202 to 204 which introduced an Apo I restriction site (AGGAACCTG to AGAAATTTG). For the second PCR reaction, the template was the PCR product of exon 8 from the patient's genomic DNA. The upstream primer for this reaction included a 5' extension of 29 bases of exon 7 sequence (position 9698 to 9726) containing the silent mutations, as well as the first 30 bases of exon 8 (positions 10543 to 10572) sequence and 15bp of insertion (lower case): (GAACTGGGAAATTTG ATCGCGGTGCTGGGCGAGCACGACCTC AGCGAGCACGACc tcagcgagcacgacGGGG). The downstream primer extended from position 10927 to 10905 within exon 8 (GGTTGCGCAGCCCTGGCCCCAGC). For the third PCR reaction, the products of the first and second reactions, and the upstream primer from reaction 1 and downstream primer from reaction 2 were used to generate the overlapping PCR fragment containing the insertion. The presence of the mutation was confirmed by Apo I digestion.

The construction of plasmid $pT7^{SalI}$-$FVIIWT^{EcoRI}$ has been described previously (17). The product of the third PCR reaction was purified, digested with Xba I and Kpn I, then ligated into this vector which had been digested with the same enzymes, to make the clone pT7- $^{SalI}FVIIMT^{EcoRI}$. Cloned inserts were sequenced. Sequencing confirmed the correct sequence including the insertion mutation.

Construction of expression vectors.

^{SalI}FVIIWT^{EcoRI} and ^{SalI}FVIIMT^{EcoRI} fragments were prepared from the corresponding pT7 vectors, and ligated into the expression plasmid, pED-mtx^{r} provided by Dr. Randal J. Kaufman. The resulting plasmids, pEDFVIIWT and pEDFVIIMT, are dicistronic mRNA mammalian expression vectors carrying the WT or MT FVII cDNAs at the 5' open reading frame and the DHFR cDNA at the 3' open reading frame(17).

Cell culture and transfection assays. For transient transfection experiments, African green monkey COS-1 cells (ATCC CRL1650) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mmol/L L-glutamine, 10 mmol/L HEPES pH 7.2, 100 units/mL of penicillin G, 100 μg/mL streptomycin and 5 μg/mL of vitamin K1 (Phytonadione, Abbott Laboratories, North Chicago, IL) in a 5% CO_2 atmosphere at 37°C. Twenty hours prior to transfection, COS-1 cells were plated on 100mm culture dishes at a density of 3 X 106 cells/dish. Fifteen μg of the pEDFVIIWT or pEDFVIIMT constructs were transfected into cells by Lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturers' instructions. After 16 h, medium was changed, and 36 h later supernatants and cell lysates were harvested and assayed for FVII:C and FVII:Ag.

To obtain stable cell lines expressing recombinant FVIIWT and pEDFVIIMT, dihydrofolate reductase (DHFR) deficient CHO cells (CHO-DUKX-B11)(18) provided by Dr. Barbara C. Furie (Boston, MA) were transfected with the pED expression vectors. These cells were grown in alpha modified essential medium (AMEM) supplemented with 10% FBS, 2 mmol/L L-glutamine, 10 mmol/L HEPES pH 7.2, 100 units/mL of penicillin G, 100 μg/mL streptomycin, 5 μg/mL of vitamin K1, 10 μg/mL adenosine, 10 μg/mL deoxy-adenosine, and 10 μg/mL thymidine. CHO-DUKX-B11 cells were plated on 100-mm culture dishes at a density of 3 X 10^6 cells/dish. Transfections were performed as described above with 15 μg of either pEDFVIIWT or pEDFVIIMT plasmid and 45 μL of Lipofectamine. Two days after transfection, cells were divided at a 1 to 8 ratio and selected for DHFR expression using medium deficient in ribonucleosides and deoxyribonucleosides. Twelve days after transfection, 14 colonies were picked at random and cultured in 12-well (24-mm) plates. At day 20 when the cells achieved confluence, each well was split into two 35-mm dishes. At 90% confluence, cell lysates were prepared and assayed for FVII:Ag by ELISA.

Metabolic labeling studies. Nearly confluent 100-mm dishes of transiently transfected COS-1 cells or stably transfected CHO cells expressing recombinant FVII were used for pulse chase experiments. Fresh media with FBS was added 4 h before cells were deprived of methionine for 30 min and labeled for 15 min with 2 mL of methionine-free AMEM (GIBCO-BRL, Gaithersburg, MD) containing 400 μCi Expre^{35}S Protein Labeling Mix (~73% L- [^{35}S] methionine and~22% L- [^{35}S] cysteine; DuPont NEN Research Products, Billerica, MA) in a 5%

CO_2 atmosphere at 37°C. A chase was then performed in 2 mL medium containing an excess of unlabeled L-methionine (GIBCO-BRL, Gaithersburg, MD) for various time periods. At each time point, medium was harvested and PMSF added to a final concentration of 1 mmol/L. Cell extracts were prepared in 600 μL ice-cold NP-40 lysis buffer (50 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1% (wt/vol) NP-40) supplemented with 1 mmol/L PMSF. The cell lysates were precleared overnight at 4° C with 100 μL of 20% (vol/vol) fixed Staphylococcus Aureus Cowan I (SAC) coupled with a rabbit anti-mouse IgG (Sigma, St. Louis, MO) in NP-40 lysis buffer. Immunoprecipitation of FVII was accomplished by incubating precleared cell lysates and conditioned media with 25 μg of monoclonal antibody MC1476 for 4 h at 4°C. The resulting immune complexes were adsorbed with 70 μL of 20% (vol/vol) Protein A Sepharose (Sigma, St. Louis, MO) coupled 5:1 (vol/vol) with rabbit anti-mouse IgG antiserum in NP-40 lysis buffer. Pellets were washed four times in NP-40 lysis buffer and resuspended either in buffer for further enzymatic digestion (see below) or in polyacrylamide gel electrophoresis (PAGE) sample buffer with or without reducing agents, and denatured by heating to 95°C for 5 min. The immunoprecipitated proteins were resolved by SDS-PAGE in 8% (wt/vol) gels, and the radioactivity incorporated into FVII bands was analyzed using a Bio-Rad model 501 PhosphorImager and radiography on X-OMAT-AR film (Eastman-Kodak Co., Rochester, NY).

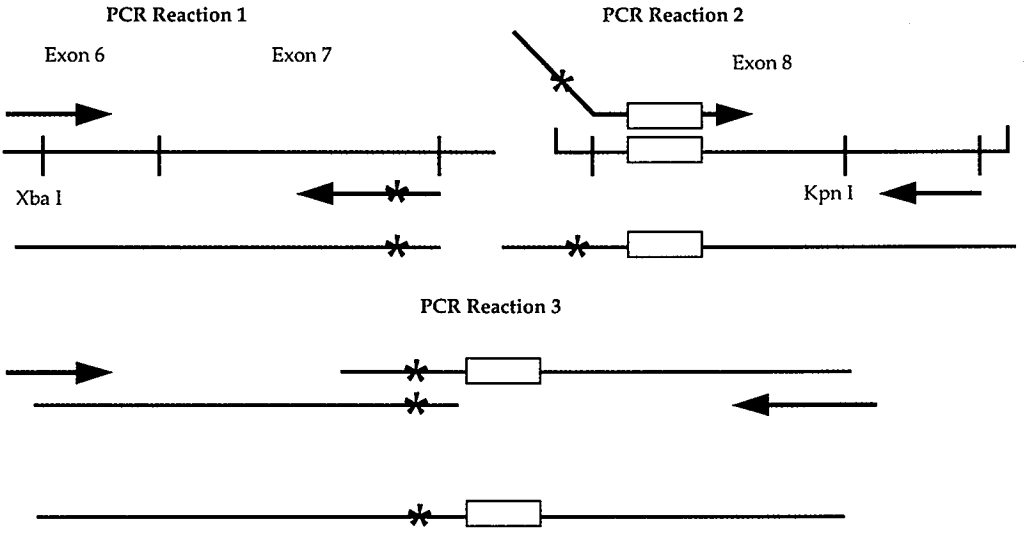
In vitro transcription/translation of FVIIWT and FVIIMT. One μg of pT7-FVIIWT and pT7-FVIIMT were linearized by EcoR I digestion and incubated in rabbit reticulocyte coupled transcription/ translation reactions (Promega Corp., Madison, WI) for 90 min at 30°C. Twenty μCi of translation grade methionine (DuPont-New England Nuclear, Billerica, MA) was included in the reactions to label the synthesized FVII protein, and a control reaction to which no plasmid DNA had been added was run in parallel. Aliquots of the reactions were electrophoresed on a 12% polyacrylamide gel under denaturing conditions.

Effect of protein degradation inhibitors on FVIIWT and FVIIMT levels. To study the effect of protein degradation inhibitors on FVII biosynthesis, confluent stably transfected CHO cells grown in 100-mm dishes were incubated with media containing ammonium chloride (50 mmol/L), leupeptin (100 μmol/L), N-acetyl-Leu-Leu-Norleucinal (50 μg/ml) or Brefeldin A (10 μg/ml) (Sigma, St. Louis, MO) dissolved according to the manufacturers' recommendations and used at previously published concentrations(19-23). After 4 h, cell lysates were harvested and assayed for FVII:Ag.

Results

Coagulation assays and genomic studies. The coagulation assays performed on the 5 year old patient from Oman revealed severe FVII deficiency with a FVII:C of less than 1% of FVII:C and FVII:Ag of 10%. The asymptomatic parents had FVII:C levels of

Fig. 1 - **Introduction of the 15bp insertion mutation into FVII cDNA by three overlapping PCR reactions.**

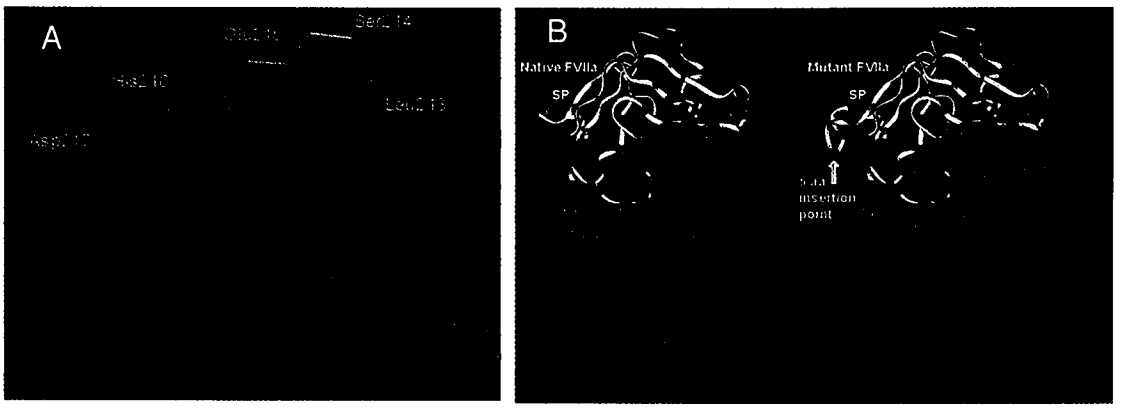


The arrows (→) for the primers show directionality, the asterisk (*) shows the introduced Apo I site and the box (▭)represents the 15bp insertion. The details of the three PCR reactions including templates, positions of the primers on FVII and restriction sites are included in text.

50% (mother) and 60% (father). To determine the molecular abnormality causing FVII deficiency in the patient, the entire coding region and 490 bp of promoter region of the FVII gene were amplified and screened by SSCP and heteroduplex analysis. An abnormally migrating fragment was detected in exon 8 by SSCP analysis. Sequence analysis of this fragment demonstrated a homozygous insertion-type mutation located at nucleotide 10554. This insertion was an in-frame 15 base pair (5 codon) duplication from Leu213 {73} to Asp217 {77} inclusive (CTC AGC GAG CAC GAC) and was located between codons 217 and 218.

Structural analysis of the mutant FVIIa. The superimposition and viewing of the crystal structure for the FVIIa-TF complex and two additional structures for unbound FVIIa (PDB codes 1dan, 1qfx and 1cvw) showed that the Leu213-Asp217 {73-77} sequence had identical conformations at a surface loop in the first subdomain of the catalytic serine protease domain. They occur within a sequence 210-EHDLSEHDGDEQSRR-224, in which 10 residues possess charged side-chains and half are buried from solvent. A network of buried hydrogen bonds and salt bridges occur within this loop, which is highly exposed to solvent and adjacent to the

Fig. 2 - **Molecular graphics views of the 15bp insertion mutation (Leu213-Asp217ins) in the FVII catalytic domain.**

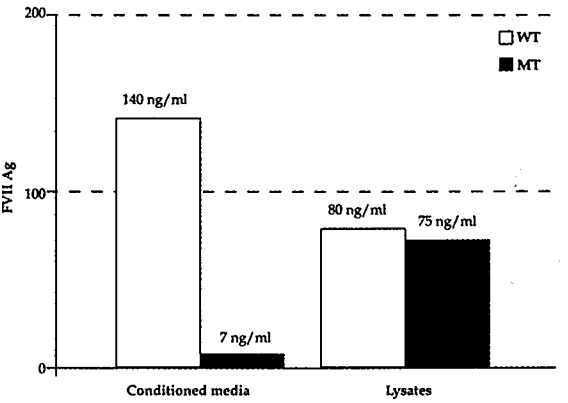


(a) In native FVIIa (PDB code 1cvw), the polypeptide mainchain backbone is shown in light blue with the exception of that for the insertion residues Leu213-Asp217 which is in yellow. The calcium ion is shown in magenta, with its six ligands being the sidechain carboxyl oxygen atoms of Glu210 and Glu220, the mainchain carbonyl oxygen atoms of Asp212 and Glu215, and two water molecules. With the exceptions of Glu210 and Glu220, the sidechains are not shown.
(b) Comparison of native FVIIa (left) with a model of FVIIa containing the 15bp insertion mutation (right) (PDB code 1dan). The two structures are shown in ribbon views, where the N-terminal and C-terminal subdomains of the catalytic domain are shown in white and magenta respectively. The EGF-1 and EGF-2 domains are shown in green, and are proximate to the two TF domains at the bottom (not shown). The insertion mutation is shown as a yellow ribbon, while the catalytic triad is represented by three red spheres on the opposite side of the domain to that of the 15bp insertion.

active site cleft between the two subdomains of the catalytic domain. The loop is followed by the long β-strand G with many buried sidechains that passes over the top of the domain surface and ends at Asp242 {102} of the His-Asp-Ser catalytic triad.(7) The insertion of five residues at this location is therefore expected to disrupt the correct formation of the loop and the insertion of β-strand G into the protein surface, and may lead to misfolded protein that may be degraded or not secreted. In addition to the possibility of misfolding, the insertion coincides with a calcium binding site formed by direct metal contacts with the sidechains of Glu210 {70} and Glu220 {80} and with the mainchain oxygen atoms of Asp212 {72} and Glu215 {75}. Two water molecules fill the two remaining calcium coordination sites (Figure 2a). All six oxygen-calcium bond lengths range between 2.1-2.4 Å (PDB code 1cvw). The insertion will directly affect the Glu215-calcium interaction, as Figure 2a shows that the correct mainchain conformation on the loop from Asp212 {72} to Glu215 {75} is critical for this calcium-binding site. It is also likely that the 15 bp insertion will disrupt the Glu220 interaction with calcium. In order to visualize the possible effects of the Leu213-Asp217 insertion on FVIIa, its sequence was added

to the crystal structure of FVIIa by homology modelling methods. This was most simply achieved by the deletion of His216 {76} and Asp217 {77}, and reorienting these two residues outwards in order to permit the subsequent addition of the five additional amino acid residues. Figure 2b showed that the loop could be readily incorporated into the FVIIa structure without steric problems. This indicated that mutant FVIIa might form a folded protein, although it was not possible to comment on the probability of this correct folding taking place.

Fig. 3 - **FVII:Ag expressed by COS-1 cells following transient transfection.**



The concentrations of FVII:Ag in culture media and lysates of cells transfected with either pEDFVIIWT (open bars) or pEDFVIIMT (filled bars) were determined by ELISA after 36 hr, as described in Methods. The values shown are the mean from 6 replicate dishes. The concentration of FVII:Ag is expressed in ng/ml.

Expression studies. To investigate the influence of the insertion mutation, the pEDFVIIWT and pEDFVIIMT vectors were expressed in COS-1 cells by transient transfection. ELISA assays of the cell lysates demonstrated that FVII:Ag levels of WT (80 ng/ml in 3 X 10^6 cells plated in 36 h) and MT (75 ng/ml in 3 X 10^6 cells plated in 36 h) were similar. However, the FVII:Ag levels in the conditioned media of MT was significantly reduced to 5% (7 ng/ml) of WT (140 ng/ml) (Figure 3). No Factor VII procoagulant activity was detectable in the conditioned media of cells transfected with FVIIMT (FVII: C<1%), consistent with the result obtained by assay of the patient's plasma. Recombinant FVIIWT , in contrast, had normal procoagulant activity that was similar to the level of FVII:Ag.

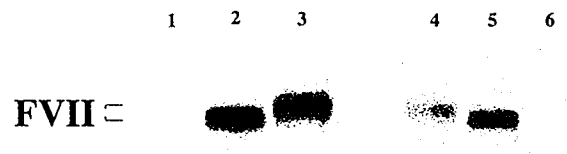
To compare the synthesis of FVIIWT and FVIIMT proteins, *in vitro* transcription/translation assays were performed. This experiment showed that, when both the mRNA and the nascent protein were protected from degradation, equivalent amount of FVIIWT and FVIIMT proteins were produced. It was noted that the FVIIMT protein, containing the additional 5 amino acids, migrated more slowly than the FVIIWT protein under denaturing conditions (Figure 4).

We then investigated FVII biosynthesis and secretion in CHO cells stably transfected with pEDFVIIWT cDNA or pEDFVIIMT cDNA. Follo-wing a 15 min pulse with ^{35}S methionine, a chase was performed at 0, 30, 60, 120 and 240 min. At each time point, the recombinant FVIIWT in cell lysates was immunoprecipitated with Mab1476.

Detectable levels of FVIIWT were maximal at 60-120 min and decreased after this time as a consequence of protein secretion.

Approximately equal amo-unts of intracellular protein appeared to be synthesized for FVIIWT and FVIIMT. (Fig.5a: lane 1-5, 6-10) No intracellular accumulation was observed for FVIIMT (Figure 5a lane 1-10). However, in the conditioned media, FVIIMT was barely detectable at 120 min (Fig.5b lane 7) and there was only a very small amount (5 to 10% of FVIIWT) after 240 min (Fig.5b. lane 8). The same difference in electrophoretic mobility between FVIIWT and FVIIMT in the transcription/ translation experiment was also present in the immunoprecipitation analysis (Fig. 5a and 5b).

Fig. 4 - **In vitro transcription/translation of FVIIWT and FVIIMT.**



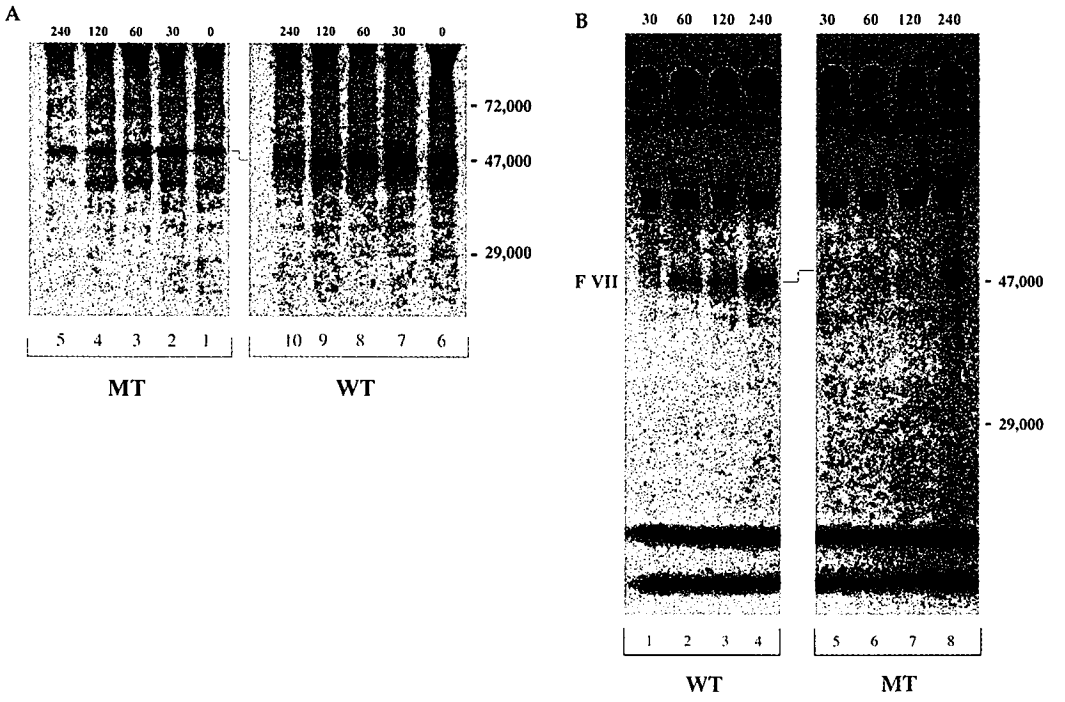
In vitro transcription and translation in a rabbit reticulocyte lysate system was done with either no added DNA template (lanes 1 and 6), or with closed circular pT7FVII plasmid (WT, lane2 and MT lane 3), or with linearized pT7FVII plasmid (WT, lane 5 and MT lane 4). Sample were analyzed on a 12% SDS PAGE under reducing conditions.

Effects of protein degradation inhibitors on FVII biosynthesis. Because metabolic labelling studies indicated that the FVIIMT protein was neither accumulating within the cell nor being secreted, we hypothesized that it might undergo intracellular degradation. We analyzed by ELISA the effects of various inhibitors of protein degradation on the intracellular FVII levels in stably transfected CHO cells (Table 1). NH_4Cl, a general inhibitor of lysosomal proteolysis, modestly increased WT FVII:Ag levels ($p=0.001$) but not MT FVII:Ag ($p=0.74$). Also treatment with leupeptin, which inhibits cathepsins B, D, H, L, did not change FVII:Ag levels significantly, demonstrating that FVIIMT is not likely to be degraded in the lysosome. We also investigated the effect of Brefeldin A on intracellular levels of FVII:Ag. Brefeldin A is a compound which blocks protein transport from the endoplasmic reticulum to the Golgi complex and causes retrograde translocation of Golgi components back to the endoplasmic reticulum. Intracellular levels of FVIIWT were increased by 50% after treatment with Brefeldin A ($p<0.0001$), but there was no significant difference for levels of FVIIMT ($p=0.12$). This result suggests that partial degradation of the mutant protein occurs in a preGolgi compartment.

Discussion

The vast majority of mutations in the FVII gene are missense mutations, but a few nonsense mutations and small deletions and insertions have also been reported(6,7). We report a novel homozygous insertion type mutation that causes a severe deficiency of FVII. The insertion was localized between

Fig. 5 - **Pulse-chase of cell lysate and conditioned media of the cells stably transfected with pEDFVIIWT or pEDFVIIMT.**



After 15 min of pulse with S35 methionine, cells were chased for 30, 60, 120 and 240 min. Equivalent amounts of cell lysate (A) and conditioned media (B) for both WT and MT constructs were immunoprecipitated using Mab1476 against FVII and analyzed by 8% SDS-PAGE under non reducing conditions. The molecular weight marker is localized at the right side of the figures.

codons 217-218 leading to a duplication of codons 213 to 217. This mutation appears to be a classic case of slipped mispairing between two copies of a direct repeat (gcg agc acg ac) separated by 4 bp in which the wild type evolved by internal duplication, thereby setting up the possibility of a mutation caused by slipped mispairing. Computer views of the 5 amino acid insertion in the FVIIa crystal structure revealed that the insertion occurs at the calcium binding site in the catalytic domain. The six calcium-oxygen ligands are the same as those reported for bovine trypsin(24). As the intracellular concentration of calcium is 1.5 mM and its plasma concentration is 2.5 mM(25), FVII is expected to interact with calcium as soon as it is synthesized. This report describes expe-

riments that characterize the first mutation directly affecting this FVII calcium binding site, and the lack of detectable procoagulant activity in FVIIMT stresses the importance of this site for the normal function of FVII.

Similar intracellular levels of FVII:Ag measured by ELISA or detected by immunoprecipitation confirmed the normal synthesis of FVIIMT. In contrast, the reduced levels of FVII:Ag observed in conditioned media supported our hypothesis of a secretion defect caused by the insertion mutation. After 240 min of chase, very small amounts of labelled FVIIMT were detected in the conditioned media.

However no intracellular FVII accumulation was seen suggesting the additional possibility of intracellular degradation. Thus, we chose to block various pathways of protein biosynthesis and transportation using NH_4Cl (an inhibitor of lysosomal degradation) and leupeptin (an inhibitor of cathepsins B, D, H and L). These inhibitors did not markedly change intracellular FVII:Ag levels. However treatment with Brefeldin A, an agent that usually blocks protein transport from the endoplasmic reticulum to the Golgi complex and causes translocation of Golgi components back to the endoplasmic reticulum, caused the intracellular level of FVIIWT to increase by 56%, whereas no significant difference was seen for FVIIMT.

The small amount of FVIIMT protein secreted by transfected cells was not proteolytically active in a one-stage coagulation assay, and this confirmed the phenotype of the patient. The lack of activity in secreted FVIIMT is well explained in terms of the direct perturbation of two calcium binding sites at the mainchain carbonyl atoms of Asp212 and Glu215. The insertion of five residues implies that the loop mainchain structure will be modified and that many of the sidechain interactions between buried residue pairs involving His216, Asp217, Asp219, Glu220, Glu221 and Arg223 will no longer be possible. Thus the insertion is expected to disrupt the sidechain positions of the two other calcium binding ligands at Glu210 and Glu220. In agreement with the modelling, alanine scanning mutagenesis has shown that mutations in the sidechains of Glu210, Asp212, Leu213 and Glu220 lead to marked decreases in proteolytic function without affecting TF binding(26). Mutants involving Glu220 reduced the coagulant activity of FVII to 0.1%(27). Even though the homology modelling shows that it was possible to add a surface loop to the surface of FVII, the correct formation of folded mutant FVIIa may be difficult to achieve for reason of the extensive sidechain interactions in this loop region. If so, this would lead to the misfolding of the catalytic domain of FVIIMT. Accordingly FVIIMT may either be secreted at lower levels or be abnormally degraded.

This explanation would account for the observed reduction in the FVIIMT:Ag level when this was measured using two monoclonal antibodies specific for the FVIIa light chain(9,12).

In conclusion, we report the first insertion

mutation in the FVII gene and the first naturally-occurring mutation to be associated with the FVII calcium binding site in the catalytic domain. This mutation is associated with various defects including abnormal folding, intracellular degradation, secretion failure and lack of detectable procoagulant activity.

Acknowledgements

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Molecular analysis of the ERGIC-53 gene in 35 families with combined factor V-factor VIII deficiency

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Abstract

Combined factor V-factor VIII deficiency (F5F8D) is a rare, autosomal recessive coagulation disorder in which the levels of both coagulation factors V and VIII are diminished. The F5F8D locus was previously mapped to a 1-cM interval on chromosome 18q21. Mutations in a candidate gene in this region, ERGIC-53, were recently found to be associated with the coagulation defect in nine Jewish families.

We performed single-strand conformation and sequence analysis of the ERGIC-53 gene in 35 F5F8D families of different ethnic origins. We identified 13 distinct mutations accounting for 52 of 70 mutant alleles.

These were 3 splice site mutations, 6 insertions and deletions resulting in translational frameshifts, 3 nonsense codons, and elimination of the translation initiation codon. These mutations are predicted to result in synthesis of either a truncated protein product or no protein at all.

This study revealed that F5F8D shows extensive allelic heterogeneity and all ERGIC-53 mutations resulting in F5F8D are "null."

Approximately 26% of the mutations have not been identified, suggesting that lesions in regulatory elements or severe abnormalities within the introns may be responsible for the disease in these individuals. In two such families, ERGIC-53 protein was detectable at normal levels in patients' lymphocytes, raising the further possibility of defects at other genetic loci.

Introduction

Combined factor V-factor VIII deficiency (F5F8D) (MIM 227310) is a rare, recessive coagulation disorder characterized by reduction in levels of both factor V and factor VIII, to less than 20 U/dL(1-4.) The severity ranges from mild (factor V and VIII levels of 10 to 20 U/dL) to moderate (levels 5 to 10 U/dL)(5). Linkage of the F5F8D locus to human chromosome 18q was reported in nine nonAshkenazi Jewish families by homozygosity mapping(6) and by classical linkage analysis in 19 families of Irani, Pakistani, and Algerian origin(7). The same locus was, therefore, implicated in different ethnic groups. The difference in clinical severity and the lack of a specific haplotype in the second group of families(7), in contrast to two distinct founder haplotypes reported by Nichols *et al*(6), suggested the existence of allelic heterogeneity, ie, that more than one mutation was responsible for the disease in this sample of families. Critical recombination events localized the F5F8D gene to an interval of approximately 1 cM, between markers D18S849 and D18S1103(7). ERGIC-53, which had been previously mapped to this region(8) and codes for a 53-kD transmembrane protein resident in the endoplasmic reticulum-Golgi intermediate compartment(9), was shown by mutation analysis to be the gene responsible for F5F8D(1). Two mutations were identified. All Sephardic Jewish patients from five families tested were homozygous for a donor splice site mutation leading to premature protein truncation, and all

patients from four Middle Eastern Jewish families had a single base pair insertion at codon 30. In Epstein-Barr virus (EBV) transformed cell lines from these patients, Western blotting and immunofluorescence analysis indicated complete absence of ERGIC-53 expression. (1) To investigate the molecular origin of this coagulation disorder we have studied 35 additional F5F8D families from different ethnic backgrounds by single-strand conformational analysis (SSCA) and sequencing of polymerease chain reaction (PCR) products of exons 1-13 and flanking intronic sequences of the ERGIC-53 gene. As expected from our previous haplotype analysis,7 numerous mutations in the ERGIC-53 gene were found to be responsible for the disease. All 13 identified discrete mutations were predicted to lead to a deficiency or absence of functional protein. These mutations accounted for a total of 52 of 70 mutant alleles (74%). In addition, a number of polymorphisms were identified, some of which result in amino acid changes.

Materials and methods

Description of families. Blood was collected from 16 families from Iran, 6 families of Pakistani origin, 8 from Italy, 2 of Chinese origin, and 1 each from Algeria, Britain, and South Africa (Table 1). Informed consent was obtained from all families. The majority of Iranian and Pakistani families were consanguineous (19 of 22) involving first cousin marriages. Factor V and factor VIII assays were performed by a one-stage procedure by using congenitally deficient plasma substrates and normal pooled plasma as reference (10). The levels of factor V:C and factor VIII:C detected in the plasma of affected individuals are shown in Table 1.

DNA isolation and PCR amplification. Genomic DNA was purified from blood leukocytes, according to standard protocols. The ERGIC-53 gene structure was determined by PCR and sequence analysis as described in another report (Nichols *et al*(11), this issue). For the mutation screening, one affected patient from each family (35 individuals) and four normal individuals were used. The ERGIC-53 gene was analyzed by PCR amplification of all 13 exons, including intron-exon junctions (with the exception of the intron 7-exon 8 junction). The primers used for the PCR amplifications (5'>3') are shown in Table 2.

Single-strand conformational analysis. Each sample was analyzed independently with either small- or large-format polyacrylamide gels: (a) PCR was performed from genomic DNA in a total volume of 15 μL, containing 2.6 pmol of each primer, 1.3 μmol/L of each dNTP, and 0.25 U Taq polymerase. After denaturation at 94°C, the amplification program consisted of 10 touchdown cycles of 30 seconds denaturation at 94°C, 30 seconds annealing between 60 and 50°C, and 30 seconds elongation at 72°C followed by 20 cycles (30 seconds 94°C, 30 seconds 50°C, 30 seconds 72°C). Ten additional cycles were then performed in a new reaction on an aliquot (2 μL) of the first PCR to reduce the amount of genomic DNA

Tab. 1 - **Identified Mutations, Geographical Origin, and Clinical Characteristics of F5F8D Patients**

Patient	Origin (Consanguinity)	Phenotype Factor V/VIII	Mutation	ERGIC-53 Protein
A1	Iran (C)	13/14	912-913insA (cd 305) 912-913insA (cd 305)	ND
A2	Iran (C)	7/7	822G>A (IVS7 DS -1) 822G>A (IVS7 DS -1)	-*
A3	Iran (C)	14/13	912-913insA (cd 305) 89-90insG (cd 30)	ND
A4	Iran (C)	10/11	IVS9 +2 T>G IVS9 +2 T>G	ND
A5	Iran (C)	5/10	? ?	ND
A6	Iran (C)	14/13	822G>A (IVS7 DS -1) 822G>A (IVS7 DS -1)	ND
A7	Iran (C)	7/7	? ?	ND
A8	Iran (C)	5/6	1214-1218delAAATG (cd 406) 1214-1218delAAATG (cd 406)	ND
A9	Iran (C)	18/8	822G>A (IVS7 DS -1) 822G>A (IVS7 DS -1)	ND
A10	Iran (C)	12/15	23delG (cd 8) 23delG (cd 8)	ND
A11	Iran (C)	18/17	89-90insG (cd 30) 89-90insG (cd 30)	ND
A12	Iran (C)	2.5/2.2	822G>A (IVS7 DS -1) 822G>A (IVS7 DS -1)	-*
A13	Iran (C)	4.6/5.5	? ?	ND
A14	Iran	10/6	? ?	ND
A16	Iran	9/13.5	R202X (CGA>TGA) R202X (CGA>TGA)	ND
A17	Iran (C)	5/7.5	R202X (CGA>TGA) R202X (CGA>TGA)	ND
A18	Pakistan (C)	10/15	K302X (AAA>TAA) K302X (AAA>TAA)	-*
A19	Pakistan (C)	14/14	R456X (CGA>TGA) R456X (CGA>TGA)	-*

Patient	Origin (Consanguinity)	Phenotype Factor V/VIII	Mutation	ERGIC-53 Protein
A20	Pakistan (C)	8/6	K302X (AAA>TAA)	-*
			K302X (AAA>TAA)	
A21	Italy	10/11	?	+°
			?	
A22	Italy	8/24	?	-*
			?	
A24	Italy	17/26	M1T (ATG>ACG)	ND
			M1T (ATG>ACG)	
A25	Italy	9/10	M1T (ATG>ACG)	-*
			M1T (ATG>ACG)	
A26	Pakistan (?)	18/18	K302X (AAA>TAA)	ND
			K302X (AAA>TAA)	
A27	China	7/9	R456X (CGA>TGA)	-*
			R456X (CGA>TGA)	
A28	UK		?	ND
			?	
A29	South Africa	11/22	?	+°
			?	
A30	Pakistan (C)	6/3	K302X (AAA>TAA)	ND
			K302X (AAA>TAA)	
A31	Pakistan (C)	14/18	K302X (AAA>TAA)	ND
			K302X (AAA>TAA)	
A33	Italy		?	ND
			?	
A34	Italy	9/27	IVS5 +1 G>T	ND
			IVS5 +1 G>T	
A35	Italy	6/23	IVS5 +1 G>T	ND
			IVS5 +1 G>T	
A36	Italy	1.4/2.7	1208-1209insT (cd 403)	ND
			1208-1209insT (cd 403)	
A37	China (?)	17/19	R456X (CGA>TGA)	ND
			R456X (CGA>TGA)	
B1	Algeria (C)		31delG (cd 11)	ND
			1delG (cd 11)	

F5F8D levels were measured as described in Materials and Methods. Western blot analysis of ERGIC-53 protein levels was as described (see Materials and Methods and Fig 3).
*Abbreviation: ND, not determined. * Indicates no ERGIC-53 protein detected. ° Indicates normal ERGIC-53 levels.*

Tab. 2 - **Primers Used for PCR Amplifications (5' > 3')**

Exon	Forward	Reverse
1	TCG CGT TCC AGA ATC CAA G	TCA GCA CAC CAG GGT AGC
2	CAG TTT GGA AAT GTA CAT TGA G	GGG AAC AGT TAG AGG CTA G
3	CTA GCC TCT AAC TGT TCC C	CTC ACA GCC TAA CTC TGT TG
4	TGT AAG TCA CTT CAT AGT AC	CAA TGT ATT TCA TAA GGA TTC C
5	TGA AAA GCT GAG TGT CTT GT	GAA AAG TGA TAC TGT AAC ATT G
6	GAA ACA AAA CTG AAT AGT AGT C	ACA AGT CTA CAT ATC CCT AA
7	AGA GTG CCA TTG CCT TTA CC	CAA ACC TAA GTT AGT CTT CC
8	CAC CAG ATA AAG AAA TTT CG	AGG CAA CAC AGA GAC TCA AG
9	CAC TTT GGT CAC TTA CGT TA	TCT ATG AGC ACA TAG TAC AG
10	GGG AAG TAA AGA AGA AGG GC	AAT CAC ATA ACA CAC AAA CG
11	GTG ATT TTA TTG TAT CAA GAG	AGT ATG AGT TCT TCC TTT CC
12	GGG GAT AGA AGG TTT TCT GG	GAA CAT AGA TAA CTT AGT TG
13	CTG TTC ATT TCA GTT CAC AT	AAT TCC CTC AAA ACG ACA TC

versus amplified product before SSCA. PCR products were denatured with an equal volume of denaturation buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue) for 10 minutes at 94°C. A 6-μL sample of each was then loaded on a 12.5% GeneGel Excel (Pharmacia Biotech, St Albans, UK). Electrophoresis was at 600 V at 12°C for 3 hours (for fragments of approximately 300 bp: ie, exons 1, 2, 6, 7, 9, 11, 12, 13) or 2 hours (for fragments of approximately 200 bp: ie, exons 3, 4, 5, 8, 10). The gels were stained by DNA silver staining (Pharmacia Biotech). SSCA variants were purified and directly sequenced with the primers used for the amplification with a semiautomated sequencer (ABI 377; Perkin-Elmer Applied Biosystems, Foster City, CA), using standard protocols. Mutant alleles of heterozygous patients were cloned by using TA cloning (Invitrogen, La Jolla, CA) and purified and sequenced as previously described. (b) PCR was performed from genomic DNA in a total volume of 50 μL of 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.8, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100 containing 150 ng of each primer, 200 μmol/L of each dNTP and 1 U of Red Hot DNA polymerase (Advanced Biotechnologies, Epsom, UK). After denaturation at 94°C for 7 minutes, 30 cycles of denaturation: 94°C, 1 minute; annealing: either 50°C or 55°C, 1 minute; extension: 72°C, 3 minutes were performed followed by a final extension at 72°C for 10 minutes. PCR products were labeled by the incorporation of α^{33} Pd-ATP (56 kBq/50 μL reaction, 377-110 Tbq/mmol; Amersham Life Science, Little Chalfont, UK). SSCA was performed as described by Michaelides *et al*(12) on a 40-cm polyacrylamide gel run at 4°C. PCR products were purified by filtration by using a Microcon 100 spin column (Amicon, Stonehouse, UK)

before direct sequencing with the thermosequenase dye terminator cycle sequencing kit (Amersham Life Science) according to the manufacturer's instructions and analyzed on an Applied Biosystems 373A DNA automated sequencer (Perkin-Elmer Applied Biosystems, Warrington, UK). The primers used for PCR and direct sequence analysis were as in Table 2, with the exception of (5' > 3') exon 1, F: TCGCGTTCCA GAATCCAAG, R:AGCA-CACCAGGGTA GCCG; exon 6, F: AGTCATAAAATGGA TCGATTG, R: TTCCCAATAAAACACAC CTC; and exon 8, F: TGTT-AACCTTTCCG TAGTGG, R: GCTAGGCAA-CACAGACT CAA.

Allele-specific PCR. The 822G > A mutation was analyzed by allele-specific PCR amplification using the following primers: (5' > 3'): GTAATCTCCTATGGAACTTTT and either the wild-type TTGAAAATATG TAAAA-TTACT or mutant TTGAAAA-TATGTTTGTAAAAT-TACC. After denaturation at 94°C for 7 minutes, 30 cycles of denaturation: 94°C, 1 minute; annealing: 53°C, 1 minute; extension: 72°C, 3 minutes were performed followed by a final extension at 72°C for 10 min. The products were analyzed by electrophoresis on 2% agarose gels.

Analysis of intragenic polymorphic markers. The G3R polymorphism was analyzed by restriction digestion of the exon 1 PCR product with *Bam*H1 (G3, *Bam*H1 site; R3, no *Bam*H1 site). Similarly, the R14Q polymorphism was analyzed by restriction digestion of the exon 1 PCR product with *EagI* (R14 *EagI* site; Q14, no *EagI* site). The R117 polymorphism was studied by SSCA of the exon 2 PCR product.

RT-PCR analysis. Polyadenylated RNA was isolated from EBV transformed lymphocytes and first-strand cDNA synthesis performed according to standard techniques (Amer-sham Pharmacia Biotech, St Albans, UK) with 200 ng of an ERGIC-53 exon 8 specific antisense primer: 5'-TTTATCCAATTCTTG-TTGAAAG-3'. Five microliters of the reaction was used for PCR amplification with an ERGIC-53 exon 6 specific oligonucleotide primer: 5'-AATGATCAATAATGGCTTTA-CA-3' and the exon 8 specific primer used for the cDNA synthesis under the following conditions: (a) Initial denaturation 95°C, 5 minutes then 25 cycles of denaturation, 94°C, 30 seconds; (b) Annealing, 57°C, 30 seconds; (c) Elongation, 72°C, 30 seconds, and (d) A final elongation step at 72°C, 10 minutes. A further 5 μL of the reverse transcription (RT) was used for PCR amplification of GAPDH by using the following oligonucleotides: 5'-T-GAGTACGTC GTGGAGTCCAC-3' and 5'-ACCAGGAAAT GAGCTTGACA-3'. The products were analyzed by 2% agarose gel electrophoresis.

Western blot analysis. ERGIC-53 protein was detected by Western blot analysis as previously described (1) on EBV transformed cell lines from affected and carrier individuals in families A18, A19, A20, A21, A22, A25, A27,and A29.

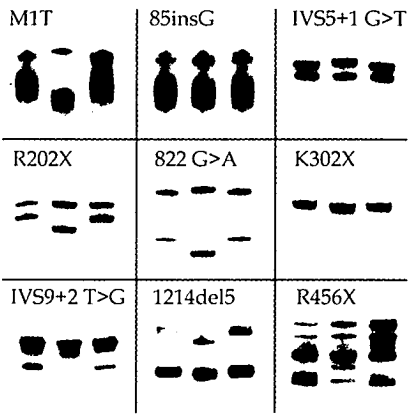
Results

A total of 35 patients, ie, one affected individual per family, were analyzed for mutations in the ERGIC-53 gene. Each of the 13 exons and the adjacent intron-exon boundaries of the ERGIC-53 gene were amplified by PCR and analyzed in two laboratories by SSCA with both small and large format polyacrylamide gels. Representative SSCA patterns of nine mutations are shown in Fig 1. In families for whom no mutation was identified by SSCA, each exon (complete with the flanking intronic sequences) was sequenced.

Many variant SSCA patterns were identified. For each discrete pattern, the appropriate fragment was subjected to nucleotide sequencing. In all cases, a particular SSCA pattern was associated with a unique sequence difference. All heterozygous sequence variants were characterized by direct DNA sequencing of both strands of the PCR products and, in some cases, the PCR products were cloned and sequenced.

A total of 13 definite mutations, representing 52 of 70 F5F8D alleles, were identified (Table 1, Fig 2). We have confirmed the high degree of allelic heterogeneity suggested by our previous haplotype analysis(7), which is in marked contrast to the founder effect observed in Jewish families by Nichols *et al*(1,6). The majority of the patients in whom mutations were identified are true homozygotes rather than compound heterozygotes (25 of 26), largely because of the high degree of consanguinity found in the parents of patients with this rare, autosomal recessive disease (Table 1).

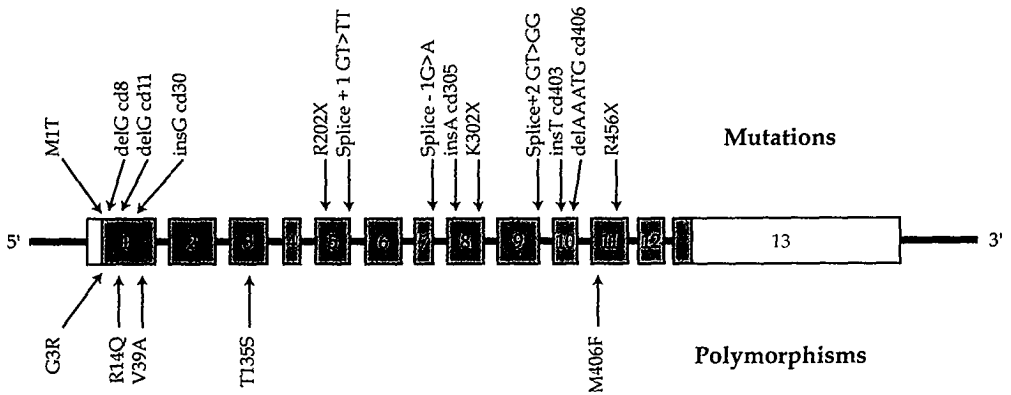
Fig. 1 - **Examples of SSCA for nine discrete mutations in ERGIC-53. SSCA was performed according to protocol 1 in Materials and Methods. In each panel, the abnormal pattern caused by the mutation is in the middle lane surrounded by two controls.**



Nonsense mutations. Three different nonsense mutations were found, in exons 5, 8, and 11, accounting for a total of 20 F5F8D alleles (27% of all mutant alleles) (Table 1, Fig 2). One of these, K302X (AAA > TAA), was homozygous in five of the six Pakistani families studied. The other two, R202X and R456X, were CGA to TGA mutations in accordance with the known CpG to TpG hypermutability(13). The R456X was found in both Chinese and Pakistani families on a different haplotype (A19 homozygous for R117R cgg; A27 homozygous for R117R cga) showing recurrence of this mutation.

Deletions and insertions. A total of six small deletions and insertions leading to disruption of the reading frame and prema-

Fig. 2 - Schematic representation of the ERGIC-53 gene showing mutations causing combined F5F8D (above the gene) and normal polymorphisms (below). Exons, indicated by rectangles, are numbered from 1 to 13 and are drawn to scale. The coding portion of the gene is shaded, with the white portion of exon 1 representing the 5' UTR and the white portion of exon 13 the 3' UTR, theexactsizeofwhichisunknown. The introns are indicated by narrow lines and are not to scale.

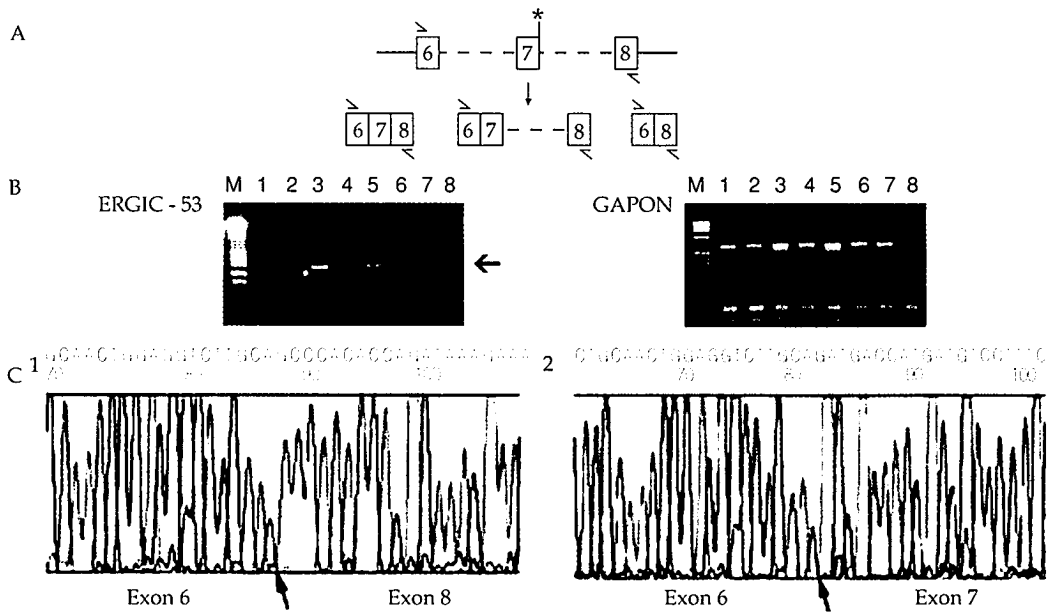


ture termination of translation were identified, on 14 different mutant alleles (Table 1, Fig 2). These were in exon 1, 23delG (in a G4 tract), 31delG (in a G3 tract), 89-90insG (in a G4 tract); in exon 8, 912-913insA (in an A8 tract); in exon 10, 1208-1209insT, and 1214-1218delAAATG (deletion of one of two copies of a repeated pentanucleotide). The 89-90insG mutation, which we found in two Iranian families, is the common mutation of Middle Eastern Jewish families(1).

Missense mutation in the initiator ATG. A mutation of particular interest was found in the translation initiation codon in two Italian families from our study (Table 1, Fig 2), and four Italian families in the report by Nichols *et al*(11). This ATG > ACG mutation is predicted to abolish translation since the next in frame ATG in the coding sequence is found in exon 6, thus leading to the complete absence of ERGIC-53, as confirmed by Western blotting.

Splice site mutations. Two different splice site mutations predicted to result in severe abnormality of RNA processing and one putative splice site mutation were identified in three different exons accounting for a total of 14 F5F8D alleles (Table 1, Fig 2). In intron 5, a G > T substitution at the invariant GT dinucleotide of the donor splice site (GT > TT) was found in homozygosity in families A34 and A35, both from Italy. In intron 9, the donor splice site invariant dinucleotide GT was mutated to GG in both alleles of the affected members of family A4 from Iran. Both of these mutations are

Fig. 3 - (A) Cartoon representation of intron 6-intron 8 of the ERGIC-53 gene. The primers used for the RT-PCR analysis are indicated by an arrow and the asterisk indicates the position of the IVS7-1 mutation. Potential RT-PCR products resulting from normal splicing, inclusion of intron 7 or skipping of exon 7 are shown. (B) Agarose gel electrophoresis of the RT-PCR products after amplification of polyadenylated RNA with ERGIC-53 exon 6-exon 8 specific primers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) specific primers. The GAPDH amplification was a control for mRNA integrity. The cDNA synthesis was primed with the exon 8 specific primer and oligo dT. Lane M, markers; lane 1, individual A2 (homozygous 822G > A); lane 2, individual A12 (homozygous 822G > A); lane 3, human endothelial cell line; lane 4, unaffected individual; lanes 5, 6, and 7, affected individuals with the M1T mutation; lane 8, no DNA control. The arrow marks the normally spliced product. The asterisk marks the incorrectly spliced product. (C) DNA sequence chromatograms of: 1, the incorrectly spliced product, resulting from skipping of exon 7, observed in RT-PCR of polyadenylated lymphoblastoid RNA from individual A12; 2, the major product observed in RT-PCR of polyadenylated RNA from a human endothelial cell line. The exon boundaries are indicated by an arrow.



expected to result in abnormal splicing of the ERGIC-53 mRNA(14). Indeed, a different mutation in the same donor splice site of intron 9 (GT > GC) was previously identified in five Sephardic Jewish families, resulting in an apparently complete block of splicing of this intron(1).

Another modification, a G > A change in the last nucleotide of exon 7 (822G > A), ie, in the -1 position of the donor splice site con-

sensus sequence of intron 7 was found in homozygosity in four Iranian families: A2, A6, A9, and A12 (Table 1, Fig 2). This mutation does not change the amino acid corresponding to the modified codon as both CCG and CCA code for proline. However, it is possible that abnormal splicing occurs at least in some mRNA molecules because the sequence surrounding the splice site is modified. We screened the DNA of 24 unaffected and the remaining 13 affected Iranians by allele-specific PCR; none carried the mutation.

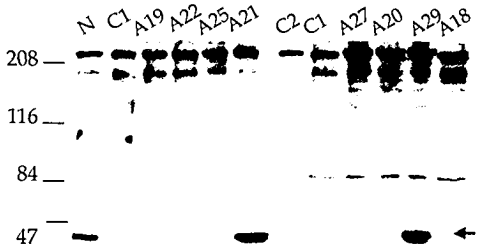
To determine the consequences of this mutation on ERGIC-53 mRNA we isolated polyadenylated RNA from lymphoblastoid cell lines from patients: A2, A12 (both homozygous 822G > A), A24 and A25 (homozygous M1T), and one unaffected individual. cDNA synthesis was primed by using an oligonucleotide corresponding to the antisense sequence of exon 8. RT-PCR between exons 6 and 8 followed by sequencing of the products confirmed that in A2 and A12, exons 6 and 8 were directly contiguous after the skipping of exon 7; no normal cDNA was present. No skipping of exon 7 was observed in the other tested individuals (Fig 3). Skipping of exon 7 leads to absence of ERGIC-53 protein in lymphoblasts (Table 1, A2 and A12).

Polymorphisms. The SSCA and nucleotide sequence analysis showed several common polymorphisms in the ERGIC-53 gene that result in amino acid substitutions. These substitutions are not associated with nor do they cause F5F8D because they were found in homozygosity in normal individuals, or in affected alleles in which another, deleterious, mutation was also present. These polymorphisms are R14Q (CGG to CAG), V39A (GTC to GCC), T153S (ACT to TCT), and M410L (ATG to TTG) (Fig 2). In addition there is a common CGA to CGG polymorphism at the Arg codon 117 that does not result in an amino acid substitution. Furthermore, a deletion of 2 Ts at nucleotides 16-17 of IVS4 was also commonly observed. These polymorphisms may be useful in linkage studies involving chromosome 18q21. In our study, these polymorphisms allowed us to confirm the compound heterozygosity (ie, two different mutations on the two ERGIC-53 alleles) observed in the Iranian patient A3 (Table 1) because this patient was also heterozygous for the R14Q and R117 polymorphisms.

In Iranian family A14, there was a nucleotide substitution G to A at codon 3 resulting in an amino acid substitution glycine to arginine. This substitution is within the hydrophobic 30-amino acid signal sequence. This substitution was not present in the DNA of any other affected individual from the Iranian sample and was not present in 24 unaffected Iranian individuals tested.

Western blot analysis of ERGIC-53 protein levels. Western blot hybridization confirmed the absence of detectable ERGIC-53 protein for two nonsense mutations (K302X, R456X) and one missense mutation (M1T), (Table 1 and Fig 4). It also showed the presence of detectable ERGIC-53 protein in individuals A21 and A29, two patients with

Fig. 4 - **Western blot analysis of ERGIC-53 protein levels in selected families with F5F8D (see Table 1 for the nature of the ERGIC-53 mutation present in each affected individual). N indicates a normal control, lanes C1 and C2 are two different mutations from reference 1 (C1: 86-89insG; C2 IVS9 + 2 T > C). The arrow indicates the position of the ERGIC-53 protein band. Normal levels of ERGIC-53 protein were also found in two unaffected family members from family A19.**



no detectable mutation in the ERGIC-53 coding sequence and flanking intron-exon boundaries.

Discussion

In this study we have analyzed the ERGIC-53 gene in 35 families with F5F8D. We have confirmed that mutations in the ERGIC-53 gene are definitely responsible for the deficiency in 74% of the families analyzed. In contrast to the two distinct founder mutations found to be responsible for the disorder in Sephardic and Middle Eastern Jewish families, we identified 13 distinct mutations accounting for 52 mutant alleles in F5F8D families of multiple ethnic origins. There were 3 different splice site mutations, 6 insertions and deletions resulting in translational frameshifts, 3 nonsense mutations, and a missense mutation in the initiator methionine. In addition we also identified several amino acid polymorphisms.

One of the splice site mutations, found in homozygosity in four Iranian families but absent in 74 other Iranian alleles tested, was situated at position -1 of the donor splice site consensus sequence of intron 7 (822G > A). At this position in the consensus donor splice site, G is found in 78% and A in only 10% of mammalian genes; therefore, we confirmed by RT-PCR analysis that this modification leads to exon skipping and loss of the open reading frame. There are 56 known G to A mutations in the 1 nucleotide of the donor splice site in human genes associated with disorders(14) (Human Gene Mutation Database; http://www.uwcm.ac.uk/uwcm/-mg/hgmd0.html, including factor V, factor VII, factor VIII, and factor IX. Abnormal splicing was studied in several of these cases and the resulting exon skipping ranged from 30% to 100%. In addition, use of cryptic donor splice sites has been reported as resulting in translational frameshift and abnormal protein(14,16).

One of the sequence differences, G3R, which was found in homozygosity in one Iranian family (A14) but was absent in 80 other Iranian alleles tested, is within the 30-amino acid signal sequence and may be either a polymorphism or a causative mutation. This position is not highly conserved. For example, the rat sequence contains valine at position 3 (Genbank no. U44129). The G3R substitution does not change the probability that the first 30 amino acids is a

signal peptide (http://psort.nibb.ac.jp; http://www.cbs.-dtu.dk/services/SignalP). However, it is now recognized that signal sequences have a more complex structure than previously anticipated, allowing for multiple and independent interactions with the translocation machinery(17) (see Note Added in Proof).

There appears to be little or no correlation between the precise mutation and the severity of the F5F8D phenotype because (a) all identified mutations are predicted to lead to an absence of mature protein and (b) some recurrent mutations were found in association with strikingly different levels of factor V and factor VIII (compare, for example, families A6 and A12 or families A26 and A30).

The human ERGIC-53 is a 53-kD transmembrane resident protein of the ER-Golgi intermediate compartment, a distinctive vesicular organelle in the secretory pathway(18). The protein is homologous to leguminous lectins, presenting mannose-selective and calcium-dependent binding(19-21). Additional ERGIC-53 homologues have been identified in the rat, *Xenopus laevis*, and *Caenorhabdi-tis elegans* (22,23) (Genbank accession no. Z81097). The importance of ERGIC-53 protein in the efficient secretion of the coagulation factors V and VIII has clearly been established by its causative role in F5F8D (data presented in this report, the accompanying report by Nichols *et al*(11), and reference 1). Factor V and factor VIII are homologous proteins that share a conserved domain structure, having derived from a common ancestor molecule, with the A and C domains of the two factors showing 40% sequence identity (24,25). Both factor V and factor VIII are subject to extensive posttranslational modification, which includes the addition of multiple oligosaccharide residues, predominantly in the B-domain. Therefore, ERGIC-53 most probably interacts with the B-domains of factor V and factor VIII via a lectin like linkage. All of the mutations in ERGIC-53 described to date are null mutations. However, there is still some factor V and factor VIII activity in the plasma of the affected individuals, suggesting that there may be several bypass mechanisms for the transport of factor V and factor VIII from the ER to the Golgi. ERGIC-53 may also be required for the secretion of many other glycoproteins whose loss is not sufficient to cause a clinically recognizable phenotype. Although we have identified approximately 74% of the mutations responsible for F5F8D in our patient sample, 26% of the mutations have not been identified despite screening by SSCA with two different experimental conditions and sequencing the entire coding sequence and intron-exon boundaries of the ERGIC-53 gene. These results are similar to those reported by Nichols *et al* (11) in the accompanying report, with no mutations found in the ERGIC-53 gene in 8 of 19 families. A number of explanations are possible for this incomplete detection. The missing mutations may be located within intronic regions that were not analyzed in the current study, leading, for example, to aberrant splicing or

other RNA anomalies, or in regulatory regions situated up to several hundred kilobases away from the ERGIC-53 gene. Such mutations could only be identified by detailed investigation of ERGIC-53 transcripts in the remaining families.

However, a number of observations strongly support an alternative explanation, which is the existence of mutations at other, currently unidentified, loci. First, in all but one of the consanguineous families in whom we have identified ERGIC-53 mutations, as expected the patients are true homozygotes. In contrast, in two of the three consanguineous (first-cousin marriage) families in whom no mutations were identified, the affected individuals are heterozygous for the ERGIC-53 gene according to intragenic and flanking polymorphisms(7) (families A5: heterozygous for R14Q and R117, and A13: heterozygous for R14Q and M410L).

Secondly, affected individuals from another two families with no identified ERGIC-53 mutations (A21 and A29) have normal levels of ERGIC-53 according to Western blotting. The existence of further loci responsible for F5F8D is currently under investigation.

Note added in proof

Immunofluorescence analysis of cells expressing introduced genes for ERGIC-53 with wild-type or mutated (G3R) leader sequences showed no difference in signal peptide cleavage or distribution of protein. Hence, G3R cannot be the cause of disease in family A14. (Hans-Peter Hauri, personal communication, Basel, Switzerland.)

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chapter 4

Therapeutic Recommendations

4.1 Fibrinogen deficiency

On the basis of our clinical experience plasma levels of 40-50 mg/dL are sufficient for normal hemostasis, because patients with higher levels do not bleed spontaneously nor after surgical trauma. In the past, cryoprecipitate was the preferred form of replacement therapy. Virucidal methods cannot be currently applied to "wet" cryoprecipitate, so that even using the most stringent screening tests this fraction carries a risk of transmission of bloodborne viruses. Fibrinogen concentrates treated with virucidal methods are commercially available and convenient to use at doses of 20-30 mg/kg body weight but are expensive (Table 1). Recently, a method has been developed that allows to add to plasma pooled from at least 2500 donors a virucidal mixture made of an organic solvent and a detergent (1). Since the application of this virucidal method to plasma-derived coagulation factor concentrates used in patients with hemophilia A and B no case of hepatitis B and C nor of HIV infection has been recorded in these patients over a period of 15 years (2). Whether this safety record also applies to solvent/detergent plasma remains to be demonstrated. It is reasonable to expect the same levels of safety towards HIV, HBV and HCV that the method provides to plasma-derived concentrates used in hemophilia A and B. However, concerns remain about non-enveloped viruses that are not inactivated by solvent/detergent, particularly the hepatitis A virus and parvovirus B19. The relatively high cost of solvent/detergent plasma (3 to 4 times that of plain fresh-frozen plasma) may be a serious obstacle for its use in developing countries use. Another virucidal method applicable to plasma is based on its illumination with visible light in the presence of a photosensitizing dye such as methylene blue (3). This method, which has the advantage of being applicable to single plasma units, has not been extensively validated by *in vivo* studies.

4.2 Prothrombin deficiency

Even though the series of Iranian patients with prothrombin deficiency is definitely the largest ever reported, our clinical experience is insufficient to make firm recommendations on treatment. The long plasma half-life of the protein (72 hours) and the relatively low levels necessary for normal hemostasis (20-30 per cent) (Table 1) make the infusion of fresh-frozen plasma the most inexpensive and safe therapeutic option, specially if plasma is virally inactivated. Factor IX complex concentrates usually contain substantial amounts of prothrombin (roughly one unit of prothrombin per unit of factor IX). Doses of 20-30 U/kg, repeated as necessary according to the type and severity of bleeding episodes, are usually adequate. These concentrates carry a risk of thrombotic complications, whereas the risk of transmission of bloodborne infections is minimal since the adoption of virucidal methods (for review, see 2).

4.3 Factor V deficiency

Factor V is the only inherited coagulation disorder that must be treated with plasma,

Tab. 1 - **Treatment of rare coagulation disorders**

Deficient Factor	Hemostatic Levels	Plasma Half-life	Recommended therapy (in order of priority)
Fibrinogen	40-50 mg/dL	3 days	- Fresh-frozen plasma (FFP) (15-20 ml/kg), preferably virus-inactivated Fibrinogen concentrate (20-30 mg/kg)
Prothrombin	20-30%	3 days	- FFP (15-20 ml/kg) Factor IX complex concentrate (20-30 U/kg)
Factor V	10-15%	36 hours	- FFP(15-20 ml/kg), preferably virus-inactivated
Combined factor V and VIII	10-15%	-	- As for factor V
Factor VII	10-15%	4-6 hours	- Factor VII concentrate (30-40 U/kg every 12 hours)
Factor X	10-15%	40 hours	- FFP (15-20 ml/kg) Factor IX complex concentrate (20-30/kg)

because no concentrate is currently available. With a half-life of 36 hours and minimal hemostatic levels of the deficient factor of 10-20 per cent (Table 1), attention must be made to avoid fluid overload when repeated infusions are needed. In our experience, single daily dosages of 15-20 ml/kg are usually adequate to control most spontaneous hemorrhages in soft tissues and mucosal tracts. A frequent symptom such as epistaxis can usually be controlled by local measures and antifibrinolytic amino acids, so that replacement therapy can be avoided. In a few surgical cases, prolonged treatment with large amounts of plasma caused fluid overload. Hence, a factor V concentrate is warranted.

4.4 Factor VII deficiency

Factor VII has a very short plasma half life (4-6 hours) (Table 1). The need of two-three daily infusions to keep factor VII above the hemostatic levels of 10-15 per cent renders difficult the treatment with plasma and warrants the use of concentrates. Virally-inactivated plasma concentrates containing almost exclusively factor VII are produced by at least two commercial manufactures (Table 2) and two daily doses of 30-40 U/kg have been successfully used by us to handle major surgery without fluid overload. Recombinant factor VIIa, originally developed and produced to bypass the defect of the intrinsic system in patients with factor VIII and factor IX inhibitors, is efficacious in patients with factor VII deficiency. However, it remain to be seen whether its viral safety justifies the use of this expensive product when less expensive virus-inactivated plasma-derived concentrates are available.

4.5 Combined deficiency of factor V and factor VIII

Since bleeding symptoms occur in patient with levels of factor V and VIII below 15 per

Tab. 2 - **Factor concentrates for rare coagulation disorders**

Brand	Company	Viral inactivation
Fibrinogen THE	Green Cross, Japan	TNBP/ polysorbate 80* plus dry heat 60° C, for 72 hr
Clottagen (Fibrinogen)	LFB, France	TNBP/polysorbate 80 plus vapor heat, 60°C for 10 hrs, 80°C for 1 hr
Factor VII (Provertin)	Immuno, Austria	Vapour heating
Factor VII	Bio Products Laboratory, UK	Dry heat, 80°C for 72 hr
Facteur VII-LFB	LFB, France	TNBP/polysorbate 80
Acset (Factor VIIa)	LFB, France	TNBP/polysorbate 80
Novoseven (Factor VIIa)	Novo Nordisk, Denmark	None
Factor XIII	Bio Products Laboratory, UK	Dry-heat, 80°C for 72 hr
Fibronogammin HS (Factor XIII)	Centeon, Germany	Pasteurization at 60°C for 10 hr

* *Solvent/detergen*

cent, it is surmised that hemostasis can be achieved by raising both factors above this level with 10-15 ml/kg of fresh-frozen plasma, preferably virally-inactivated.

4.6 Factor X deficiency

Factor X has a relatively long plasma half-life of 40 hours (Table 1). Hence, fresh-frozen plasma can be conveniently used to treat relatively mild bleeding episodes which usually necessitate one or two infusions of 15-20 ml/kg to reach and maintain plasma factor levels of 10-15 per cent. Another possibility is to use factor IX concentrates, which contain factor X and are virally inactivated. The units of factor X contained in concentrates correspond roughly to those of factor IX. Usually 20-30 U/kg every other day are sufficient to handle major surgical operations.

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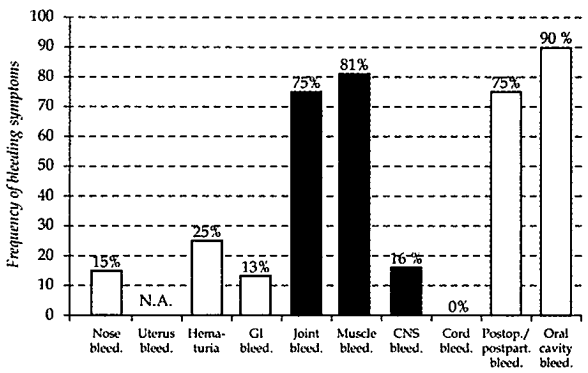
chapter 5

General Conclusion

5.1 Pattern of bleeding symptoms

Evaluation of the bleeding symptoms found in this large series of patients suggests that in general rare coagulation disorders tend to be less severe than the hemophilias caused by factor VIII or IX deficiency. This views is substantiated by the comparative analysis of symptoms occurring in 150 patients with hemophilia A strictly matched with those with factor V, VII and X deficiencies for age and severity (three-fourth had severe disease). As expected, among hemophiliacs the most prevalent symptoms were joint, muscle and post-operative hemorrhages, whereas bleeding in the mucosal tracts was relatively rare (Figure 1). Among patients with rare coagulation disorders, only a minority had spontaneous hemarthroses and muscle hematomas, in spite of the fact that three-fourth of them has severe deficiencies of clotting factors. Accordingly, permanent damage to the musculoskeletal system and the resulting handicaps was rarer. There were other differences in the type and frequency of bleeding symptoms (Figure 2-7). Hematuria, not rare in hemophilia, was relatively frequent only in factor X deficiency but occurred very rarely or not at all in the remaining defects. Bleeding from the umbilical stump, not seen in hemophiliacs and thought to be typical of the inherited defects of fibrin formation (afibrinogenemia and factor XIII deficiency), was not unusual also in prothrombin, factor V and factor X deficiency. A mild bleeding symptom such as epistaxis, rare in hemophiliacs, was very frequent in all rare defects. In afibrinogenemia and factor V deficiency this mucosal-type symptom might be explained by a concomitant platelet defect of these factors, reflected by a prolonged bleeding time. There is however no obvious explanation for the frequency of nose bleeds in defects of coagulation factors such as prothrombin, factor VII and X, not contained in platelets and not directly involved in primary hemostasis. The same considerations apply to another mucosal-type symptom such as menorrhagia, a frequent event that often caused iron deficiency in women. There was no clear evidence that the coagulation defects reduced fertility in affected women, nor did they cause recurrent abortions. The only exception is afibrinogenemia that adversely affects implantation of the embryo

Fig. 1 - **Factor VIII deficiency**



(1) and is sometime associated with recurrent abortions (2,3). As it could be expected, surgical procedures were often followed by excessive bleeding when carried out without adopting preventive measures. Hemophilia was originally described in the Talmud because it caused fatal bleeding at birth after circumcision. Among Muslims circumcision is usually practiced at a later age (2-3 years) than in Jews, so that bleeding after this procedure was not always the revealing symptom in rare coagulation disorders. Life-endangering hemorrhages in the gastrointestinal tract and central nervous system were rare. There are several examples in the literature of the frequent occurrence of these manifestations in patients with rare coagulation disorders, but report bias is likely to have emphasized their prevalence. The total number of patients with rare coagulation disorders studied by us is still relatively limited, although they are a large proportion of those listed in Iranian registry. More importantly, patients were not selected on the basis of the severity of bleeding symptoms as it has probably occurred in many cases previously reported in the literature.

At the moment, the observed quali-

Fig. 2 - **Fibrinogen deficiency**

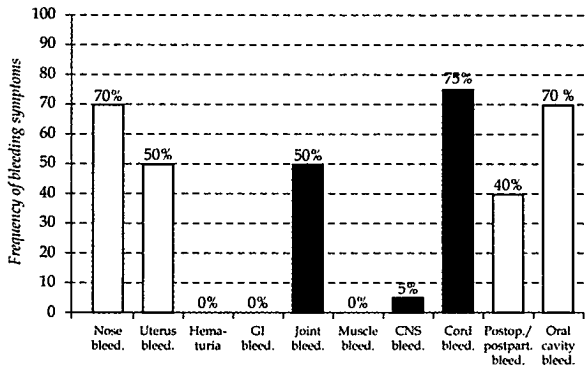


Fig. 3 - **Prothrombin deficiency**

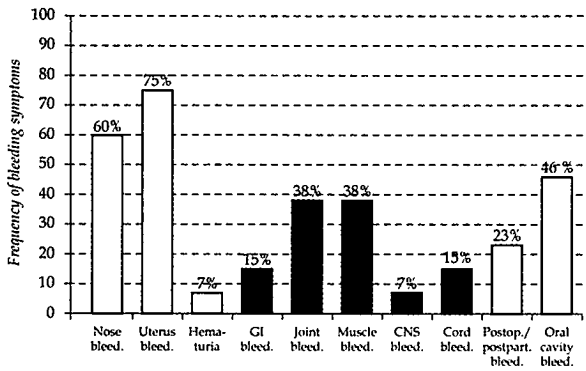


Fig. 4 - **Factor V deficiency**

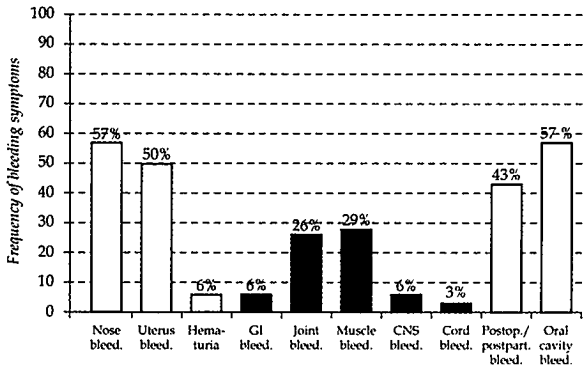


Fig. 5 - **Factor VII deficiency**

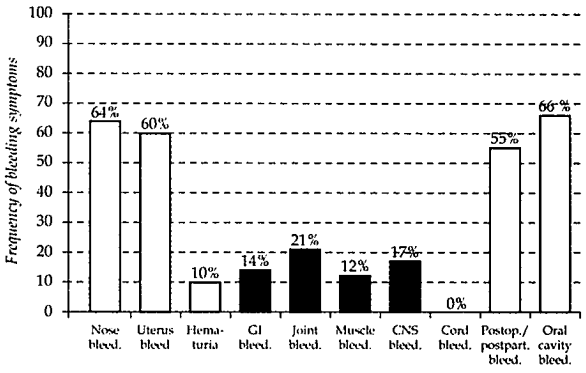


Fig. 6 - **Factor V+VIII deficiency**

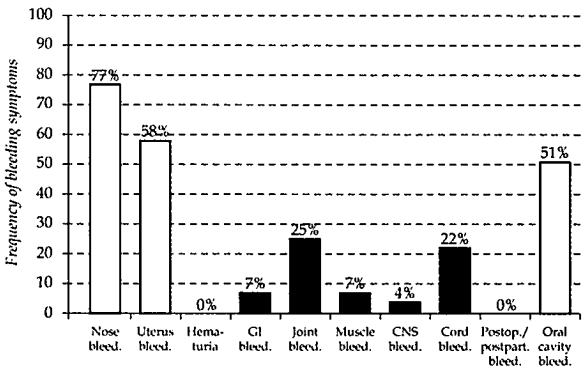
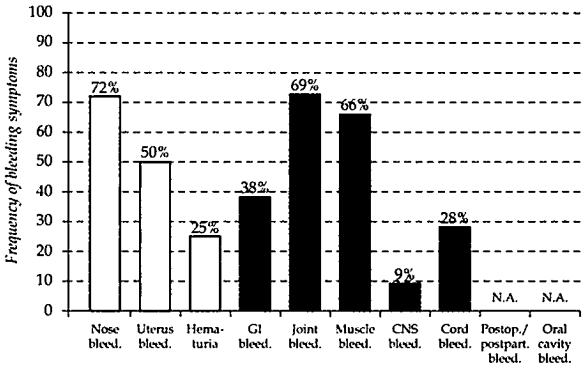


Fig. 7 - **Factor X deficiency**



tative and quantitative differences between the different defects cannot be easily explained. Animal models of gene knock-out indicate that bleeding is more severe and often lethal in mice rendered deficient in prothrombin, factor V and factor X than in those with factor VII and fibrinogen deficiency (1,4,5,6,7). Even though this pattern of varied severity of factor deficiency in mice is roughly similar to that observed in our patients, one has to consider that in knock-out models the gene encoding a given clotting factor is completely abrogated. Accordingly, no trace of RNA or protein is produced, whereas the gene lesions found so far in humans are usually less devastating and less likely to completely impede protein production. Due to the limits of sensitivity of the bioassays of clotting factors currently used, it is difficult to establish whether or not a patient with unmeasurable plasma levels has a totally absent production of a given clotting factor. Hence, it is unclear to which extent mouse results apply to humans. Perhaps more understanding of the variability of bleeding symptoms in different defects will be possible when our knowledge on the spectrum of gene mutations will be larger than now.

5.2 Molecular defects

Paper 3.1 analyzed 27 patients with factor VII deficiency, particularly from the Middle East. In these patients we used SSCP analysis to verify gene abnormalities in the coding region and in the 5' and 3' untranslated regions. Cases with abnormal bands on SSCP have been subsequently analyzed by direct sequencing. Nineteen mutations were found in this group of patients, 12 novel and 7 already reported. Conformational analysis of the crystal structure of the factor VIIa-tissue factor complex was used in the attempt to explain the effect of these mutations. The results of this study significantly enlarge our knowledge on gene mutations associated with factor VII deficiency and expand by 50% the number of the homozygous mutations already reported the current available database (1997)

Paper 3.2 reports for the first time a homozygous (-2bp) deletion mutation in the preproleader sequence of factor VII. This mutation is important because leads to a complete absence of measurable factor VII in plasma, conserving only 9 aminoacids of the N-terminal preproleader sequence followed by a short series of irrelevant aminoacids.

Our results indicate for the first time that a complete factor VII deficiency does not necessarily result in fatal hemorrhage immediately after birth, at variance with what observed in the factor VII knockout mice (6) and in another family with complete absence of factor VII (3). It remains to be demonstrated how in our patient the coagulation defect is partially compensated for in vitro.

Paper 3.3 evaluates through expression studies the mechanism responsible for severe FVII deficiency in homozygous Italian patients with the Gly97Cys or the Gln100Arg mutations in the second EGF domain of FVII. The severe clinical phenotype of the patient with Gln100Arg is consistent with our data demonstrating a major secretion defect as well as markedly impaired function of the small amount of factor VII that is released from cells. In the patient with Gly97Cys it was not possible to obtain sufficient FVIICys97 protein to carry out detailed biochemical studies and probably the resulting alteration in protein folding causes factor VII Cys97 to undergo degradation in a preGolgi compartment.

Paper 3.4 reports the first case of an insertion type mutation in the factor VII (FVII) gene that results in severe FVII deficiency with FVII coagulant activity (FVII:C) of <1% and factor VII antigen levels (FVII:Ag) of 10%. DNA analysis revealed a homozygous 15 bp in-frame insertion type mutation at nucleotide 10554 within the catalytic domain of FVII. This insertion consists of a duplication of the residues Leu213 to Asp217 (Leu,Ser,Glu,His,Asp), probably arising by slipped mispairing between 2 copies of a direct repeat (GCGAGCACGAC) separated by 4 bp. The importance of the Asp -212 and Leu-213 codons in factor VII structure and their

integrity was studied by Dickinson *et al* using alanine scanning mutagenesis(8). They report that the mutations affecting proteolytic function are mapped on the face of the factor VIIa protease domain. Functional defects were observed for the replacements of residues within the catalytic cleft and most probably structural effects were seen for Asp-212 (c72) and Leu-213 (c73). Considering the importance of these residues in the proteolytic function any disruption created by the insertion mutation may effect the stabilization of the activity of protease domain in this mutant protein.

Molecular graphics analyses of FVIIa showed that the insertion is located at the surface of the catalytic domain in an exposed loop stabilized by extensive salt-bridge and hydrogen bond formation at which the calcium binding site is located. The insertion may interfere with protein folding during FVII biosynthesis and/or diminish procoagulant activity through the loss of calcium binding.

To explore these two hypotheses, wildtype FVII (FVIIWT) and mutant FVII (FVIIMT) cDNAs were expressed transiently in COS1 cells and stably in DHFR deficient Chinese hamster ovary cells (CHO). In lysates of cells transfected with either the FVIIWT or FVIIMT constructs, the FVII:Ag levels were equivalent. However the amount of FVII:Ag secreted by cells transfected with FVIIMT was 5-10% of that secreted by cells transfected with FVIIWT. Using stably transfected CHO cells, pulse chase studies demonstrated that FVIIMT did not accumulate intracellularly. A part of this recombinant protein was degraded in PreGolgi compartment as demonstrated by using different inhibitors of protein degradation. Accordingly, only small amounts of FVII were secreted into conditioned media, with undetectable procoagulant activity.

These results verify both the hypotheses derived from inspection of the FVIIa crystal structure, and demonstrate that both a secretion and a functional defect is the mechanism whereby this insertion causes FVII deficiency.

Paper 3.5: In the only study published so far on the molecular basis of combined factor V and factor VIII deficiency, mutation in the ERGIC 53 gene were found in nine Jewish families(9).

In the collaborative study reported in paper 3.5 we screened the gene using SSCP analysis in 35 new families of different ethnic groups and identified 13 different mutations accounting for 52 of 72 mutant alleles. These were 3 splice site mutations, 6 insertions and deletions resulting in translational frameshift, 3 nonsense codons. The mutations are predicted to result in the synthesis of either a truncated protein product or no protein at all. In our study, mutations have not been identified in approximately one fourth of the families studied, suggesting that other still unidentified lesions beside those in the ERGIC 23 gene are the molecular basis for the combined deficiency of factor V and factor VIII.

5.3 Recommendations on treatment

Our recommendations are summarized in (Table 1: Chapter 4.) The avoidance of transmission of bloodborne infectious agents is the primary requisite in the choice of replacement material. Cost is the next most important determinant. Fresh-frozen plasma is inexpensive and has the advantage of containing all coagulation factors. Every effort should be made to use plasma that has undergone a procedure of viral inactivation. Virally-inactivated factor concentrates are available for the treatment of several disorders (Table 2: Chapter 4) and should be preferred when virally-inactivated plasma is not available or repeated infusions causing fluid overload are needed, as it may occur at surgery or in cases of bleeding in the central nervous system. For a few disorders, such as factor V and combined factor V and VIII deficiency, no concentrate is currently available, so that fluid overload may occur when large amounts of plasma are infused. Hence, manufacturers of plasma products should consider to produce factor V-containing concentrates. In factor VII deficiency concentrates are essential, because the half-life of this factor is so short (ca. 6 hours) that closely spaced infusions of more than two-three doses of plasma one likely to create fluid overload. In prothrombin and factor X deficiency factor IX complex concentrates can be used for treatment. There is no obvious need for specific factor concentrates, even though the unnecessarily high post-infusion levels of vitamin-K dependent coagulation factors might be one of the causes for thrombogenicity of these concentrates(10).

Prevention of rare coagulation disorders through prenatal diagnosis is feasible in couples who have already had affected children, provided the mutation under-lying the deficiency is known. Primary prevention might be achieved by discouraging consanguineous marriages. Even though the cultural, religious and economic roots of this practice are deep in some communities in Iran, consanguineous marriages are becoming much less frequent in large cites and among younger generations.

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chapter 6

Summary

Clinical symptoms. The type of clinical manifestations and optimal treatment are not well established for recessively bleeding disorders, that have prevalences in the general population varying between 1:500,000 (factor VII deficiency) to 1:2,000,000 (prothrombin deficiency). These prevalences increase 8 to 10 times in countries where consanguineous marriages are customary. To establish the type and severity of symptoms in each coagulation defect, 237 patients with the inherited deficiencies of fibrinogen, factor II, factor V, combined factor V and factor VIII and factor X have been investigated. The most severe symptoms were found in patients with factor X and prothrombin deficiencies, with a relatively high frequency of joint and muscle bleeding. Severe bleeding manifestations like those in the gastrointestinal tract and central nervous system were relatively rare for all defects. Umbilical cord bleeding, typical of afibrinogenemia and factor XIII deficiencies, were relatively frequent also in prothrombin, factor V and factor X deficiencies. Mucosal type bleeding symptoms such as epistaxis was relatively frequent in fibrinogen and factor V deficiencies.

Gene mutations. We report the genetic alteration of 21 families with factor VII deficiency from predominantly Middle-East countries, where this deficiency has been poorly studied. Using screening techniques as SSCP and heteroduplex analysis of PCR products and sequence analysis of the abnormal fragments we identified 19 mutations of which 12 were novel and 7 have been previously reported. Of the previously reported mutations, those at Arg152, Arg304 and Thr359 involved a CpG dinucleotide that provides an explanation for identical mutations in diverse populations. However, for the Cys310Phe mutation present in both the Iranian and Italian population haplotype studies established the possibility of identity by descent. Of the 12 novel mutations, 9 were missense mutations, localized mostly in the catalytic domain but also in the Gla domain and EGF2 domain of factor VII. Also 3 novel and one previously reported splice sites mutations were identified in these patients. We found a novel homozygous (-2bp) deletion type mutation on pre-proleader sequence of factor VII gene in 5 year old Chinese boy with severe factor VII deficiency. This mutation leads to a complete lack of detectable plasma FVII reporting that this situation is not incompatible with human life. We report also the first case of an insertion type mutation in the factor VII gene that caused a severe plasma deficiency in a 5 year old girl from Oman with factor VII: C coagulant activity of less than 1% and factor VII antigen levels of 10%. This insertion consists of a duplication of codons 212 to 217, probably by slipped mispairing between 2 copies of a direct repeat (GCGAGCACGAC) separated by 4bp. In vitro study of the mutant recombinant protein by stable cell line using DHFR deficient CHO cells revealed a combined defect, i.e., intracellular degradation

in the preGolgi compartment associated with a secretion defect demonstrated by pulse-chase labelling experiments using ^{35}S methionine. Only small amounts of FVII with not detectable procoagulant activity were secreted into conditioned media.

These results verify both the hypothesis derived from molecular graphics analysis of FVIIa, and demonstrate that both a secretion and a functional defect is the mechanism whereby this insertion causes FVII deficiency. This mutation probably does not interfere with FVII synthesis, but is associated with various defects including abnormal folding, intracellular degradation, secretion failure and loss of coagulant activity. To our knowledge, this is the first instance of a FVII deficiency caused by a perturbation at its calcium-binding site in the catalytic domain.

On the whole, the novel factor VII gene mutations identified in this study extended by 30% the data base mutation analysis of the factor VII gene.

The spectrum of mutations in the ERIGC-53 gene associated with the combined deficiency of factor V and factor VIII was substantially enlarged by this study conducted in 35 families. All the 13 mutations identified are likely to results in the production of a truncated protein or no protein at all. Our study has also shown that some patients with the combined deficiency produce normal amount of ERGIC-53, indicating that other as yet unidentified molecular defects are the basis of combined factor V and factor VIII deficiency.

Management. The treatment of rare coagulation defects is usually simpler than that of the hemophilias, because of the bleeding tendency is less severe, lower hemostatic levels of deficient factor are needed and factor half-lives are usually long except for factor VII. The first requirement of replacement therapy is safety from transmission of bloodborne infectious agents and the second requirement is low cost, particularly in developing countries. Fresh frozen plasma is inexpensive has the advantage of containing all coagulation factors and can be virally inactivated with solvent/detergent.

The research work described in this thesis is a result of four years of collaboration between four International Centres: the Haemophilia Centre of Tehran, the Hemophilia and Thrombosis Centre Angelo Bianchi Bonomi of the University of Milan, Italy, the Hemostasis Unit and Hemophilia Centre , Royal Free Hospital, University College, London and the Veteran Administration Hospital, Harvard University, Boston.
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Flora Peyvandi was born on 29 November 1964 in Iran. In 1984, she moved to Italy where in March 1985 she attended the Faculty of Medicine at the University of Milan. She obtained the MD degree on October 1991. She attended the residency in Haematology at the Department of Medicine of the Ospedale Maggiore, University of Milan, under the supervision of Prof. Mannucci. In 1996 she obtained the speciality in Hematology at this University. In November 1996 she went back to Iran to organize the collection of samples of patients with rare bleeding disorders as a material for a PhD study. She left Italy in 1996 for the United Kingdom to start a research project on the molecular characterization of FVII and FX deficiency under the supervision of Dr. David Perry at the Royal Free Hospital, University College, London, as a first step of her PhD thesis. She worked two years in this Centre to investigate the molecular alterations in 21 Iranian families with FVII deficiency. To further characterize the molecular alterations found in London she moved to Boston, VA Hospital, Harvard University where she studied the mechanisms causing FVII deficiency using the molecular and cell culture techniques under the supervision of Drs. Ken Bauer and J. Carew. In one year time of research she characterized six of the novel mutations on FVII gene using in vitro expression analysis and recombinant protein production followed by biochemical characterization. In May 1999 she came back to the Angelo Bianchi Bonomi Centre, University of Milan, Ospedale Maggiore to finish her PhD studies on the rare bleeding disorders and to continue a new line of research on molecular and cell biology studies on the serine proteases of coagulation.